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The *Anopheles* innate immune system in the defense against malaria infection

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Abstract

The multifaceted innate immune system of insects is capable of fighting infection by a variety of pathogens including those causing human malaria. Malaria transmission by the *Anopheles* mosquito depends on the *Plasmodium* parasite's successful completion of its lifecycle in the insect vector, a process that involves interactions with several tissues and cell types as well as with the mosquito's innate immune system. This review will discuss our current understanding of the *Anopheles* mosquito's innate immune responses against the malaria parasite *Plasmodium* and the influence of the insect's intestinal microbiota on parasite infection.

Keywords

Anopheles; innate immunity; Plasmodium; malaria; mosquito

Introduction

Malaria, caused by the *Plasmodium* parasite, affects approximately 3 billion people worldwide each year. The major vector for *P. falciparum* in sub-Saharan Africa is the female *Anopheles gambiae* mosquito. Given the lack of an effective vaccine against *Plasmodium* and the increased resistance of this parasite to the current arsenal of drugs and of *Anopheles* mosquitoes to insecticides, the development of novel control strategies is crucial to reducing malaria transmission [1]. Studies exploring the mosquito's innate immune defense against *Plasmodium* may contribute towards the development of such preventive and control strategies. In this review, we will discuss recent findings from studies investigating anti-*Plasmodium* defenses in the mosquito, with a specific focus on those involved in parasite elimination in the midgut.

Plasmodium infection of the *Anopheles* mosquito

Plasmodium transmission requires that the parasite complete an intricate replicative cycle in the mosquito that involves transitions through several developmental stages and interactions with the mosquito's midgut and salivary gland tissues as well as the hemocoel. This journey takes approximately 2–3 weeks (the time varies for different *Plasmodium* species and strains) and begins when the female mosquito ingests a blood meal infected with *Plasmodium* gametocytes. The male and female gametocytes develop into male microgametes and female macrogametes, respectively, in the midgut lumen. Fertilization of the gametes results in the formation of zygotes. The zygotes then transform into motile

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ookinetes that invade and migrate across the midgut epithelium, roughly 18–36 hours after the ingestion of an infected blood meal. The route of ookinete invasion across the midgut epithelium as well as the cellular responses of the midgut epithelium to ookinete invasion are still topics of controversy despite numerous studies involving diverse *Plasmodium*-mosquito combinations [2–4]. However, these responses generally involve apoptosis and an extrusion of ookinete-invaded midgut epithelial cells into the midgut lumen [5–12]. Once the diploid ookinete has reached the basal side of the midgut epithelium, it transforms into an oocyst and undergoes several rounds of replication by means of sporogony. Approximately 10–12 days after the blood meal, each oocyst contains thousands of haploid sporozoites, which are then released into the mosquito hemocoel at about 14 days after the blood meal and migrate through the mosquito hemolymph in order to invade the salivary glands. During the next blood meal, these *Plasmodium* sporozoites are injected with the saliva into the human (or another vertebrate) host, thereby completing the sexual cycle of *Plasmodium* within the mosquito vector [13,14].

Mosquito immune signaling pathways in the defense against *Plasmodium*

In order to continue its cycle of transmission and eventual infection of the human host, the malaria parasite engages in a series of complex interactions with the mosquito vector. Parasite numbers are limited by several major bottlenecks that occur in the mosquito such as when the ookinete traverses the midgut epithelium prior to the development of the oocysts on the basal side and during the migration of sporozoites to the salivary glands. [15–19]. The mosquito's innate immune system has been shown to play a key role in killing parasites and thereby affecting parasite development [20,21]. The two major arms of the insect innate immune response are: 1) a humoral response involving, for examples, a complement-like system and the transcriptional upregulation of small cationic antimicrobial peptides (AMPs) and other immune effectors and 2) a cell-mediated response that includes phagocytosis and/or melanization. Other defenses include oxidative and nitric oxide-mediated killing mechanisms.

As earlier mentioned, the innate immune system of *Anopheles*, the mosquito's main line of defense against parasites, fungi, bacteria, and viruses, is engaged at multiple stages of *Plasmodium* infection [13,22–24]. Three major signaling pathways contribute to anti-*Plasmodium* defense: the Toll, the immune deficiency (Imd), and the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathways (Fig. 1). Considerable insight into these innate immune pathways has been gathered from studies conducted in *Drosophila* [24,25].

The Toll and Imd pathways

The mosquito's anti-*Plasmodium* and antibacterial defenses are largely controlled by the Toll and Imd NF-κB immune signaling pathways (Fig. 1). The Toll pathway is primarily elicited by Gram-positive (G+) bacteria, fungi, and *Plasmodium*. Studies have also implicated this pathway in the defense against viruses [26]. The Imd pathway is elicited by Gram-negative (G−) and G+ bacteria and *Plasmodium* [22,27].

Infection-responsive activation of the Toll and Imd pathways via the recognition of pathogen-associated molecular patterns (PAMPs) ultimately leads to the nuclear translocation of the NF-κB transcription factors Rel1 and Rel2, respectively (Fig. 1). These transcription factors are negatively regulated in the cytoplasm by Cactus and Caspar, respectively. Activation of the Toll and Imd pathways allows the Rel factors to enter the nucleus and transcriptionally activate immune effector genes such as antimicrobial peptides (AMPs) and other factors. The four main classes of AMPs are defensins, cecropins, attacin, and gambicin. These AMPs act against G- and G+ bacteria, yeast, fungi, and *Plasmodium*.

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Actually, gambicin was among the first anti-*Plasmodium* factors identified [28]. Additionally, it has been demonstrated that both the Rel1 and Rel2 transcription factors can induce the expression of the AMP genes *Cecropin 1*, *Defensin 1*, and *Gambicin 1* [29].

The Imd pathway-controlled transcription factor *Rel*2 gene produces a full-length form (*Rel*2-F) that includes the carboxyl-terminal ankyrin (ANK) and death domains as well as a shorter form (*Rel*2-S) lacking such domains due to alternative splicing. The *Rel2*-S form is constitutively translocated to the nucleus, where it regulates the transcription of immune genes [27] (Fig. 1).

While the Toll pathway has been shown to be more effective in the defense against the rodent *P. berghei* parasite, the Imd pathway has emerged as the most effective pathway in the defense against the human malaria parasite *P. falciparum* [30–33]. Specifically, activation of the Imd pathway by the gene silencing, via RNAi, of *Caspar* (a suppressor of the Imd pathway) results in an Imd pathway-mediated immune defense that confers almost complete refractoriness to *P. falciparum* in three major *Anopheles* malaria vector species: *An. gambiae*, *An. stephensi*, and *An. albimanus*. In contrast, activation of the Toll pathway by the silencing of *Cactus* (a suppressor of the Toll pathway) results in a significantly greater resistance to infection with the rodent malaria parasite *P. berghei* [31]. These two *Plasmodium* species elicit diverse innate immune responses at the gene transcript level [34]. A diverse repertoire of anti-*Plasmodium* immune effectors regulated by the Imd pathway, including APL1, TEP1, LRRD7 (APL2), FBN9, and LRIM1 have been identified and studied with regard to their antiparasitic action and will be discussed later [27,33–39]. It has also demonstrated and characterized the potency of the Imd pathway in anti- *Plasmodium* defense through the use of genetically modified immune-enhanced *Anopheles* mosquitoes that express blood meal-inducible *Rel*2 in both the midgut and fat body tissues [40]. The transient activation of this transgene resulted in almost complete resistance to the human malaria parasite at a negligible fitness cost, prompting further investigation of this system as an innovative malaria control strategy [40].

The potency of the Imd pathway in the anti-*P. falciparum* response has warranted further molecular dissection in light of recent studies [31,32,40]. There are, for example, only a few studies detailing the regulation of Rel2 once it has been translocated to the nucleus. Recent work has demonstrated that the transcription factor Caudal (Cad) is an antagonist of Rel2 (see Fig. 1) and also a negative regulator of the Imd pathway's anti-*P. falciparum* defense in the *Anopheles* mosquito [41]. Cad was previously identified as a negative regulator of the Imd pathway in adult *Drosophila* [42].

RNAi-mediated silencing of *Cad* specifically compromised *P. falciparum* development in the gut tissue, suppressed the midgut microflora, and enhanced resistance to systemic bacterial infections, most likely by causing an increased transcriptional abundance of AMPs and other effector genes. Interestingly, *Cad* gene silencing resulted in increased longevity in the female adult mosquitoes, but the silencing of *Cad* impaired the mosquito's fecundity and fertility [41], indicating that Cad may display functional diversity in terms of immunity, development, and perhaps other processes.

Another study has implicated the transcriptional mediators Kohtalo (Kto) and Skuld (Skd) as participants in the regulation of the Imd pathway's anti-*P. falciparum* defense in *An. gambiae*. Depletion of the *Kto* and *Skd* genes by RNAi in the mosquito resulted in an increased susceptibility to bacterial and human malaria parasite infection, but not to infection with the rodent malaria parasite *P. berghei* [43].

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The JAK-STAT pathway

Little is known about the role of JAK-STAT in insects; however, in *Drosophila*, this pathway is involved in a variety of developmental processes. It has also been implicated in antibacterial and antiviral defense in *Drosophila* and the *Aedes* mosquito [22,44,45]. Recent studies in *Anopheles* have also linked this pathway to anti-*Plasmodium* defense [46–48]. In *Drosophila,* this signaling pathway is initiated by the binding of the cytokine ligand Unpaired (UPD) to the transmembrane receptor Domeless (DOME), leading to the phosphorylation of DOME by the JAK tyrosine kinase Hopscotch (HOP) (Fig. 1). Phosphorylation of DOME recruits a STAT, which is then phosphorylated, dimerized, and translocated to the nucleus, where it transcriptionally upregulates immune effector genes. This pathway is tightly regulated by proteins such as the suppressor of cytokine signaling (SOCS) and the protein inhibitor of activated STAT (PIAS). SOCS is transcriptionally activated by this pathway as part of a negative feedback loop that regulates STAT signaling by preventing STAT phosphorylation. PIAS inhibits signaling by binding to STAT proteins and targeting them for degradation [49,50]. There are two STAT genes in *An. gambiae* (*STAT1*/*AgSTAT-B* and *STAT2*/*AgSTAT-A*), and only one in *Drosophila* (*Stat92E*); a one-to-one orthology relationship exists for JAK and DOME in these two species [24,51] (Fig. 1).

The JAK-STAT pathway mediates immunity against the malaria parasite through both STAT genes, *AgSTAT-A* and *AgSTAT-B. AgSTAT-A* is an ancestral gene regulated at the mRNA level by the *AgSTAT-B* gene. *Ag*STAT-A has recently been shown to mediate the transcriptional activation of nitric oxide synthase (NOS), which is induced in response to *Plasmodium* infection and leads to high levels of reactive nitric oxide (NO), thereby diminishing parasite development. *Ag*STAT-A also activates the transcription of SOCS. Silencing of *AgSTAT-A* increases mature oocyst development in *P. berghei* and *P. falciparum*-infected mosquitoes [46]. These findings suggest that the JAK-STAT pathway regulates NOS expression and induces immunity to the later oocyst stages of *Plasmodium* in the *An. gambiae* midgut. However, Bahia and colleagues have recently shown that the JAK-STAT pathway controls the early stages of infection with *P. vivax*, another virulent form of human malaria, in the Brazilian malaria vector *Anopheles aquasalis* [47].

While the Toll, Imd, and JAK-STAT are the best characterized pathways, however other pathways have also been shown to play key roles in antiplasmodial immunity such as the insulin/insulin growth factor-1 (IGF-1) signaling (IIS) pathway. The activation of the IIS pathway increases susceptibility to *P. falciparum* in *An. stephensi* and may even alter NF-κB-dependent immunity [52–54].

Anopheles molecular immune responses to *Plasmodium* infection

The past 20 years have witnessed great progress in understanding the mosquito's immune system, and a variety of putative immune genes/effectors have been implicated in the defense against *Plasmodium* (reviewed in [22,51]). In particular, ookinete invasion of the midgut epithelium by different *Plasmodium* species results in the elicitation of both common and diverse molecular responses [7,34,55]. These global transcriptomic analyses have identified a plethora of genes that were later shown to represent key players in anti-*Plasmodium* defense.

One of the first anti-*Plasmodium* factors studied was the hemocyte-specific thioester complement-like protein TEP1, which binds to, and mediates killing of *P. berghei* ookinetes. TEP1 is upregulated 24 hours after ingestion of either *P. berghei*- *or P. falciparum*- infected blood and plays a role in the defense against both *Plasmodium spp.* [31,34,40,56]. Two leucine-rich repeat (LRR) proteins, LRIM1 and APL1C, are factors that function with TEP1 to regulate *Plasmodium* loads in the mosquito. Together, these three

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factors establish a complement-like pathway that is pivotal for antiplasmodial defense [39]. This defense mechanism is discussed in greater detail later in this review.

In *An. gambiae*, the superfamily of LRR domain-containing proteins is a gene family that encodes secreted, membrane-bound or cytoplasmic proteins with diverse functions; LRR immune proteins (LRIM) are members within this superfamily and have been shown to be prominent players in the antiplasmodial response[36,57]. LRIM1 is upregulated in *An. gambiae* after infection with *Plasmodium* [34]. Additionally, LRIM1 is a key antagonist of *P. berghei* and causes a substantial majority of the ookinetes to be killed while traversing the midgut, before oocyst formation [21,36]. Two other LRIM family members, *Anopheles Plasmodium*-responsive leucine-rich repeat 1 (APL1) and LRRD7, have also been shown to be involved in the defense of both *P. falciparum* and *P. berghei* development in the mosquito [21,34,35,58].

Interestingly, a recent study has shown that the *APL1* locus encodes three genes, *APL1A*, *APL1B*, and *APL1C*, which share more than 50% identity at the amino acid level [35]. This locus lies within a quantitative trait locus (QTL) that confers vector resistance to *P. falciparum* in wild mosquito populations in Africa [17,58–60]. Rottschaefer and colleagues recently examined the molecular genetic variation in the *APL1* locus in diverse West African collections of *An. gambiae*, and they found that the *APL1* locus is extremely polymorphic [37]. Within these paralogs, the *APL1A* gene was thought to be involved in the defense against *P. falciparum* through the Imd pathway. In contrast, the same study indicated that the gene *APL1C* protects the mosquito against the rodent malaria parasites *P. berghei* and *P. yoelii* only through the Toll signaling pathway [33]. Another study of the *APL1* genes showed that they behaved differently than reported by Mitri and colleagues [32]. While the role for *APL1* genes in limiting *P. falciparum* infection was confirmed, a significant role for the *APL1A* gene in the anti-*P. falciparum* immune response was not apparent. However, silencing of *APL1B* and *APL1C* had a significant impact on *P. falciparum* infection. As earlier stated, the *APL1* gene family has exhibited a complex sequence evolution, including an exceptionally high degree of polymorphism [37]. Therefore, although the latter study confirms a role for *APL1* gene family members during *P. falciparum* infection, the differences between the two studies may be explained by different versions of *APL1* sequences in the used mosquito strains, or the fact that different *P. falciparum* parasite genotype resulting in different infection intensities were used in the two studies.

Another class of *Plasmodium* effectors is the c-type lectins (CTL). Two members of this family, CTL4 and CTLMA2, are present in the hemolymph of *An. gambiae* and their transcripts are both upregulated 24 hours after blood-feeding on *P. berghei* infected mice [21]. Interestingly, these two CTLs can protect the rodent *Plasmodium* ookinetes from destruction [21]. CTL4 is also induced by *P. falciparum*-infected blood with non-invading ookinetes, while CTLGA3 is induced by invading *P. falciparum* ookinetes [34]. CTL4 and CTLMA2 are soluble proteins that are secreted in the hemolymph in the form of a disulfide-linked heterodimeric complex (similar to the LRIM1/APL1C complex which will be discussed later in this review) and protect the mosquito from infection by G- bacteria [61]. This mode of action may provide a link between their role in antibacterial defense and the melanization of *P. berghei*.

Components of the lipid transporting system, such as apolipophorin and apolipoprotein D precursors, also have a significant impact on *Plasmodium* development [34,62,63]. An apolipophorin precursor, RFABG, is induced by *P. berghei* invasion [62], and the transcript level of the apolipoprotein D (APOD) gene is increased upon *P. falciparum* infection [34]. Apolipophorin-III (ApoLp-III) has recently been identified as a player in midgut antiplasmodial defense. ApoLp-III mRNA is strongly expressed in the *Anopheles* midgut

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upon *P. berghei* infection; in addition, silencing of the *ApoLp-III* gene significantly increases *P. berghei* oocyst levels [63]. Work by Rono and colleagues demonstrated that lipophorin (Lp) reduces the parasite killing efficiency of TEP1; however the absence of Lp increased TEP1's efficiency to bind to *Plasmodium* ookinetes [64].

The fibrinogen-related proteins (FREPs) are a pattern recognition receptor (PRR) family that also exhibits anti-*Plasmodium* activity. The *FREP* gene family is significantly expanded in *An. gambiae*, with 58 members, as compared to 37 members in the mosquito *Aedes aegypti* and only 14 in *D. melanogaster* [65–68]. RNAi-mediated gene-silencing assays have indicated that the *FBN8*, *FBN9*, and *FBN39* genes are involved in the anti-*Plasmodium* defense; their involvement is specific, with FBN39 regulating only the mosquito's resistance to the human malaria parasite, and FBN9 and FBN8 being induced in response to both *P. berghei* and *P. falciparum* infection [34,65,69,70].

The G- bacteria-binding proteins (GNBPS) represent another PRR family that is important in antimalarial defense. GNBPB3 and GNBPB4 are only upregulated after challenge with *P. berghei*, and GNBPB1 is induced only by *P. falciparum*-infected blood [34,71]. Also, within the class of PRRs in *An. gambiae* are the splice variants of the *An. gambiae* Down syndrome cell adhesion molecule gene (*AgDscam*), which has been shown to protect mosquitoes against challenge with either *P. berghei* or *P. falciparum* [72,73]. The *AgDscam* gene has been identified as a hypervariable PRR with the potential to generate 31,000 alternative splice forms that are responsible for different pathogen interactions and specificities. Specifically, the Imd and Toll pathways mediate *Ag*Dscam-mediated species-specific defenses against *Plasmodium* and bacteria by regulating the alternative splicing of this gene [73]. The Imd pathway-controlled immune-responsive splicing factors Caper and IRSF1 regulate *Ag*Dscam splicing and influence anti-*Plasmodium* defense specificity. Imd pathway activation was also shown to enhance the association of *Ag*Dscam with *P. falciparum* ookinetes in the mosquito midgut epithelium [73].

Anti-*Plasmodium* defense mechanisms

The "Time Bomb" Theory

Midgut invasion by the *Plasmodium* ookinete does not leave the mosquito unharmed. According to the "Time Bomb" theory, a model of the cellular and molecular response of the *An. stephensi* midgut epithelium to *P. berghei* ookinete invasion, invading ookinetes inflict irreversible damage on the midgut epithelial cells as the parasite moves in order to reach the basal lamina, where it differentiates into an oocyst [8,74]. The invaded cells upregulate NOS expression, have fewer microvilli, undergo DNA fragmentation, and possess abnormally shaped nuclei and a remodeled actin cytoskeleton. In addition to causing cellular damage and eliciting molecular responses, the ookinetes also secrete the Pbs21 surface protein and PbSub2 protease, which may help facilitate the motility of the ookinete as it glides across the epithelium. The defense response of elevated NOS expression (and consequent NO generation) and the initiation of cell death and protrusion create a ticking time bomb and an altogether hostile environment for the traversing ookinete. Thus, the ookinete must move quickly from the damaged cells in order to continue its development in the mosquito midgut. While the majority of studies have been conducted in *An. stephensi*, NOS expression is known to be elevated in *An. gambiae* after *P. berghei* infection [20,75]. Biochemical studies in *An. gambiae* reveal nitration in *Plasmodium*-invaded midgut cells to occur as a two-step process in which the induction of NOS expression is followed by peroxidase activity [76,77]. Recent work identified heme peroxidase 2 (HPX2) and NADPH oxidase 5 (NOX5) as mediators of nitration in the *An. gambiae* midgut epithelium and demonstrated that epithelial nitration and TEP1-mediated lysis work sequentially to target

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Plasmodium ookinetes. The authors propose that nitration of ookinetes in the midgut promotes the subsequent activation of the mosquito complement system [78].

Work conducted by Shiao and colleagues proposes a wound-healing response mechanism to dead or dying ookinetes that has been argued to be in conflict with the "Time Bomb" theory [79]. In this study, the authors claim that while the majority of *P. berghei* ookinetes are killed in the extracellular space in *An. gambiae*, dead or dying ookinetes are surrounded by a polymerized actin zone formed at the basal layer of adjacent midgut epithelial cells. The formation of this zone is strongly linked to the activation of the melanization response (which is discussed later in this review). Furthermore, the study identified two factors controlling the formation of the actin zone and subsequent activation of melanization: the transmembrane receptor frizzled-2 (Fz2) and the guanosine triphosphate-binding protein cell division cycle 42 (Cdc42). Discussed later in this review, RNAi-mediated silencing of these two factors did not affect ookinete survival. Collectively, these results suggest a separation of parasite killing from subsequent reactions manifested by actin zone formation (in this case, the activation of melanization) [79].

The mosquito complement system

The complement cascade in the *Anopheles* hemolymph has emerged as a key antiplasmodial defense mechanism. As previously discussed, the mosquito complement C3-like protein TEP1 binds to the surface of midgut-invading ookinetes and marks them for killing [38]. TEP1 circulates in the mosquito hemolymph as a full-length protein and a processed form, $TEP1_{cut}$. Recent studies have independently revealed that the *An. gambiae* LRR proteins LRIM1 and APL1C are circulated in the hemolymph as a disulfide-linked heterodimer [36,39]. This complex interacts with and stabilizes $TEP1_{cut}$ and is required for TEP1 accumulation on the ookinete surface. These results reveal that the LRIM1/APL1C/$TEP1_{cut}$ complex functions as a complement-like system for parasite killing. They also indicate a potential role for the LRIM1/APL1C complex in binding multiple targets, as mammalian multi-subunit receptors have similarly been shown to robustly activate the complement pathway. The conformational changes in the LRIM1/APL1C complex can then facilitate the recruitment of additional cascade components such as TEP1-activated proteases. A recent paper has suggested that a cleaved form of TEP1 can act as a convertase for the activation of other TEP1 molecules and that the LRIM1/APL1C complex may regulate the formation of this TEP1 convertase [80]. Future in-depth studies of this complex will provide more detailed insight into complement activation and its role in *Plasmodium* killing.

Hemocyte-mediated defenses

Insect blood cells known as hemocytes (which are macrophage-like) play a key role in the mosquito innate immune response against pathogens and exist in the insect's open circulatory system. These cells function in defense against pathogens either directly through phagocytosis or indirectly through secretion of effectors such as AMPs, complement-like proteins, and effectors of the melanization response [81]. The hemolymph of the *An. gambiae* adult female contains three hemocyte sub-types: granulocytes, oenocytoids, and prohemocytes. These types can be distinguished from one another by morphological and functional markers. Granulocytes function in phagocytosis, oenocytoids play a role in melanization, and the prohemocytes are hypothesized to serve as hematopoietic progenitors [82]. Work by King and Hillyer has identified a novel type of hemocytes, known as periostial hemocytes, which surround the heart in order to phagocytose bacterial and *Plasmodium* pathogens as they flow in the hemolymph, highlighting the interaction among the mosquito innate immune and circulatory systems [83].

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Transcriptomic profiles of adult female *An. gambiae* hemocytes following bacteria and *Plasmodium* infection revealed pathogen-specific signatures of gene regulation and expression. Particularly, 4,047 genes were expressed, with 959 genes being differentially expressed following bacteria or *Plasmodium* challenge [84]. In addition to varied transcriptomic profiles, the number of circulating hemocytes in adult mosquitoes change in response to infection as well as age and physiological state [85–88]. It has been speculated that such changes may be due to a release of sessile hemocytes (hemocytes attached to tissues) or differentiation of the prohemocytes [82,86,87,89,90]. A recent study that investigated the *in vivo* distribution of hemocytes in adult *An. gambiae* demonstrated that the increase and proliferation of circulating hemocytes following infection is primarily due to mitosis in the circulating hemocytes rather than the differentiation of a progenitor cell type [91].

Interestingly, the differentiation of hemocytes has been implicated in facilitating innate immune memory in *An. gambiae.* It is much understood that the innate immune system is unable to establish memory in a fashion similar to the adaptive immune system (which is not present in insects). However, memory-like responses, termed immune priming, have been described in insects (as well as other invertebrates) [92–96]. Recent work has demonstrated an immune priming mechanism in mosquitoes in response to *Plasmodium* in the presence of their midgut microbiota. Particularly, this memory was shown to be primed by the invasion of the mosquito midgut by ookinetes. Ookinete invasion resulted in a long-lasting increase in granulocytes and enhanced immunity to bacteria. This enhanced antibacterial immunity indirectly reduced *Plasmodium* parasite survival upon reinfection [86].

While the three sub-types of hemocytes are much agreed upon, the number of circulating hemocytes within the adult mosquito is still a source of debate. For example, the authors of the aforementioned study based their conclusions on mosquitoes containing an estimated range of 30,000–50,000 circulating hemocytes [86]. However, other studies have determined that the range of circulating hemocytes in adult mosquitoes and *Drosophila* flies is between 1,000 and 5,000 [82,85,87–89,91,97]. Such discrepancies may provide an impetus to further investigate the basic aspects of hemocyte biology in addition to the methods employed to isolate and count hemocytes.

Melanization in the anti-Plasmodium defense response

Melanization is another innate immune response in the mosquito that the *Plasmodium* parasite may face. This innate immune mechanism has been genetically mapped to three QTL in *An. gambiae*, collectively called the *Plasmodium* encapsulation genes: *Pen1*, *Pen2*, and *Pen3* [98,99]. Melanin formation in the mosquito is a result of the proteolytic activation of prophenoloxidase (PPO) to phenoloxidase (PO), induced by a cascade of CLIP serine proteases. PO then oxidizes tyrosine and 3, 4-dihydroxy phenylalanine (DOPA) to form reactive quinones that produce melanin. When a pathogen invades the mosquito, the mosquito deposits melanin, which then crosslinks proteins and forms a capsule around the parasite (reviewed in [100]). The melanization process is highly regulated by serine protease inhibitors, or serpins (SRPNs), which block the activation of PO (reviewed in [101]).

Genetically selected refractory (R) and susceptible (S) strains of *An. gambiae* have provided valuable insight into the mosquito's melanization mechanism. The R strain is highly efficient at blocking *Plasmodium* development in the midgut via melanization, when compared to the S strain. We will briefly highlight the use of R and S mosquito strains in providing insight about the roles of CLIP serine proteases in melanization.

Silencing of the *CLIPA8* gene in R and in S mosquitoes in which the anti-*Plasmodium* gene *CTL4* has also been silenced has demonstrated that this CLIP protease is essential for

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activating the PO cascade and hence necessary for the melanization of *P. berghei* ookinetes [102]. Recent work by Yassine and colleagues has also demonstrated the importance of CLIPA8 in the melanization response against the entomopathogenic fungus *Beauveria bassiana* in *An. gambiae* mosquitoes [103]. CLIPA2, A5, and A7 suppress melanization, with CLIPA2 and CLIPA5 acting synergistically to block ookinete invasion. Two CLIPBs, CLIPB14 and CLIPB15, are also involved in the killing of *Plasmodium* ookinetes and participate in the defense against G- bacteria [104]. CLIPB3, B4, B8, and B17 promote ookinete invasion [101,102], and silencing of the *SRPN2* gene increases melanization and reduces the ability of *P. berghei* ookinetes to invade the midgut epithelium [105]. While depletion of the *SRPN2* gene was shown to negatively affect the ability of the parasite to invade the midgut epithelium and develop into oocysts, gene silencing of *SRPN2* in *An. gambiae* mosquitoes originally from Cameroon was not found to influence the development of field strains of *P. falciparum* [106]. The results of this study suggest that some strains of the parasite are efficient at evading the mosquito's innate immune system.

One study has recently demonstrated that CLIPB9 acts as a PPO-activating proteinase that is inhibited by SRPN2. It also showed that CLIPB9 and SRPN2 not only interact to form a regulatory unit of melanization but also affect the life span of adult female mosquitoes [107]. Another SRPN, SRPN6, mediates the defense against malaria parasites and bacteria. In particular, *SRPN6* gene expression is induced upon infection with *E. coli* and both rodent and human malaria parasites and is specifically expressed in midgut cells invaded by ookinetes and in surrounding hemocytes. Silencing of *SRPN6* in *An. gambiae* has demonstrated that its role in parasite clearance is to inhibit melanization in order to promote parasite lysis [108]. Additionally, silencing of *SRPN6* also reduces sporozoite numbers in the salivary glands [109]. A recent study has shown that the LPS-induced TNFα transcription factor (LITAF)-like 3 (LL3) in *An. gambiae* is capable of modulating *SRPN6* gene expression to influence its anti-*Plasmodium* response [110].

In addition to the aforementioned melanization effectors, other molecules and anti-*Plasmodium* factors also modulate the mosquito's melanization response. In R females, dead *Plasmodium* ookinetes have been shown to associate with a zone of actin in nearby midgut cells and with melanin deposition on the ookinete surface [79]. As discussed earlier in this review, the genes *frizzled-2* (*Fz2*) and *cell division cycle 42* (*Cdc42*) are required for these two processes of actin polymerization and melanization [79]. However, RNAi-mediated silencing of these two genes does not affect the killing of *Plasmodium* ookinetes; rather, these two factors contribute to the mosquito's wound healing mechanism during *Plasmodium* infection. Additionally, gene silencing of *CTL4* and *CTLMA2* resulted in increased ookinete melanization [21].

TEP1 may also play a role in *Plasmodiu*m melanization, since the RNAi-mediated silencing of *TEP1* renders R females unable to melanize *P. berghei*, thereby making them susceptible to infection [38]. However, silencing *TEP1* in S mosquitoes increased the number of developing parasites. The results from this work suggests that TEP1-dependent parasite killing is followed by a TEP1-independent clearance of dead parasites by lysis and/or melanization [38]. TEP1 has also been implicated in the melanization response to fungal infection in *An. gambiae* mosquitoes and Sephadex beads [103,111].

Silencing of the complement-like system genes *LRIM1* and *APL1C* also results in a decrease in melanized *Plasmodium* parasites [36]. Work by Warr and colleagues [111] has also indicated that the silencing of *LRIM1* and *TEP1* (as earlier mentioned) compromises the mosquito's ability to melanize Sephadex beads, whereas silencing of *CTL4* and *CTLMA2* did not affect bead melanization.

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Recent work has shown that some strains of *P. falciparum* are able to evade this complement-like system (i.e., TEP1, LRIM1, and APL1C) in *An. gambiae*. In particular, this work demonstrated that the silencing of *TEP1*, *LRIM1*, and *APL1C* in *An. gambiae* prevented the melanization of the Brazilian *P. falciparum* 7G8 line. However, there was no effect on infection intensity when the African *P. falciparum* strain NF54 was used, suggesting this line is able to evade this complement-like system. When *An. gambiae* R mosquitoes were co-infected with 7G8 and another African *P. falciparum* strain, 3D7, mixed infections comprised of both live and encapsulated parasites were produced in the midgut, suggesting that survival is parasite-specific in nature [112]. Silencing of *Rel2* and *PGRP-LC* led to melanization of *Plasmodium* in the mosquito midgut, suggesting that the Imd pathway is a negative regulator of the melanization response in the mosquito [27,30,113].

Mosquito – bacteria interactions

The mosquito midgut microbiota

The presence of bacteria in the midgut (the midgut microbiota) stimulate a basal innate immune activity consisting of the induction of AMPs and other immune-specific genes that act against *Plasmodium* and prime the mosquito for infection [114]. In this study, mosquitoes possessing their midgut microbiota were also shown to have upregulated key antibacterial and anti-*Plasmodium* factors, whereas mosquitoes treated with antibiotics, that eliminate the majority of the midgut microbiota, did not show this upregulation profile and were more susceptible to *Plasmodium* infection. Co-feeding mosquitoes with bacteria and *P. falciparum* gametocytes also resulted in the elicitation of an immune response and resistance to infection. Work by Meister and colleagues has suggested that the PRR molecule known as long peptidoglycan recognition protein C (PGRP-LC), which activates the Imd pathway in the mosquito in response to bacteria, modulates *Plasmodium* infection by controlling the microbial flora in the mosquito midgut [113]. Numerous surveys of mosquito midgut microbiota in laboratory and wild mosquitoes have been performed, and common bacterial genera (*Asaia*, *Enterobacter*, *Pseudomonas*, *Pantoea*, and others) have been identified, with some of these bacteria being closely associated with *Anopheles* mosquitoes. For example, the acetic acid bacteria *Asaia* has emerged as an important symbiont of *Anopheles* [115]. However, it is not clear if *Asaia* can directly reduce *Plasmodium* infection.

Several studies have shown that the mosquito midgut microbiota negatively affect the ability of *Plasmodium* parasites to develop to the oocyst stage in the mosquito gut tissue [1,116–119]. A number of bacterial species have also been shown to produce potential antimalarial compounds [120], but the effects on mosquito-stage *Plasmodium* development have not yet been examined. Bacteria may play an indirect role in parasite interference through the induction of an anti-*Plasmodium* immune response in the midgut, as discussed earlier.

Recent work by Kumar and colleagues (2010) has revealed a peroxidase/dual oxidase system that forms a dityrosine network in the midgut and decreases the permeability of the midgut to immune activators, protecting the microbiota and also providing a safe environment for *Plasmodium* to develop in the midgut [121]. Dual oxidase (Duox) is a transmembrane protein that produces the hydrogen peroxide substrate for peroxidase. RNAi-mediated silencing of the heme peroxidase-immunomodulatory peroxidase (IMPer) gene has been shown to result in decreased bacterial load in the midgut and induced the upregulation of key antibacterial effectors such as cecropin and PGRP-LB. *IMPer* gene silencing also reduced *P. berghei* ookinete and oocyst development via the induction of NOS in antibiotics-treated (also called aseptic) *An. gambiae* female mosquitoes; in addition, through the induction of NOS, RNAi-mediated silencing of the *IMPer* gene also reduced the development of *P. falciparum* in *An. stephensi* and *An. gambiae* females possessing their microbiota as well in females with decreased microbiota load via treatment with antibiotics.

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RNAi-mediated silencing of the *Duox* gene also reduced *P. falciparum* in *An. gambiae* via NOS induction. Hence, this complex when intact appears to block midgut immune responses to bacteria and *Plasmodium*, allowing proliferation and development.

Although the absolute mechanism by which bacteria inhibit *Plasmodium* is as yet unclear, their potential usefulness as a biologically based control strategy is apparent. A recent study has demonstrated engineered mosquito midgut microbiota potential as a control strategy. In this study, the investigators developed a strategy to engineer symbiotic bacteria to deliver antimalarial effector molecules to the midgut lumen, thereby rendering the mosquitoes resistant to *Plasmodium* infection [122].

Antibacterial effectors

As earlier noted, the mosquito employs antibacterial effectors to battle the malaria parasite. We have discussed some of these effectors earlier in this review with regards to their antiplasmodial roles. In this section, we will briefly highlight some of these effectors' roles in the antibacterial response.

The complement-like protein TEP1 is involved in the bacterial phagocytosis response and has been shown to bind to both G− and G+ bacteria [123]. The mosquito pathogen recognition receptor (PRR) *Ag*Dscam is a determinant of resistance and bacterial phagocytosis and also modulates the mosquito's response to *Plasmodium* infection [72,73]. Fibrinogen-related protein (FREP) FBN9 interacts with G− and G+ bacteria and appears to form dimers in order to specifically bind to bacterial surfaces with different affinities [65]. This FREP may use a multimerization mechanism similar to that of LRIM1/APL1C (earlier discussed in detail) and may dimerize with other FREPs, thereby providing diverse PAMP interaction specificities, as a means of increasing the mosquito's PRR repertoire. Whether FBN9 also forms dimers when binding to human and rodent malaria parasites remains unknown; however, direct interaction is thought to occur, as implied by FBN9's interaction with bacteria [65]. The PRR GNBPB4 is known to interact with a wide range of pathogens. Particularly, GNBPB4 has been shown to directly interact with *E. coli* and co-localize with *P. berghei* ookinetes [124].

Two other immune-responsive factors involved in the mosquito's antibacterial and antimalarial responses are the *Rel2*-S and *Rel2*-F isoforms of the *Rel2* gene. These isoforms not only modulate the defense against G- and G+ bacteria but also regulate several of the AMPs and antiparasitic genes, as mentioned earlier in this review. The immunoglobulin superfamily (IgSF) members known as the infection-responsive with immunoglobulin domain (*IRID*) genes are factors that also participate in the mosquito's antibacterial and antiplasmodial responses, with the *IRID6* gene functioning to limit *P. falciparum* as well as bacterial infection [125]. Lysozymes, another class of antimicrobial immune effectors, are also important in the antiplasmodial defense. Lysozyme c-1 (LYSC-1) has recently been shown to act as a protective agonist of the development of *P. berghei* and *P. falciparum* oocysts. This antimicrobial effector binds directly to *Plasmodium* oocysts following midgut invasion in *An. gambiae* [126,127]. In addition, silencing of the *LYSC-1* gene in *An. gambiae* as well as in the Asian malaria vector *An. dirus* significantly reduced *P. berghei* infection [127,128].

Concluding Remarks

The *Anopheles* mosquito makes use of many weapons to battle *Plasmodium*. The molecular and cellular events involved in the infection of a mosquito with different *Plasmodium spp.* may be quite similar yet also divergent, indicating the great complexity and intricacy of parasite- mosquito interactions. Given the increasing prevalence and spread of malaria,

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especially in Africa, there is an impetus for further dissection of the innate immune system of *Anopheles*, with an emphasis on how it modulates and regulates *Plasmodium* infection. The insight and knowledge gained from such studies can provide the necessary tools for creating antimalarial strategies based on amplifying the mosquito's anti-*Plasmodium* defenses.

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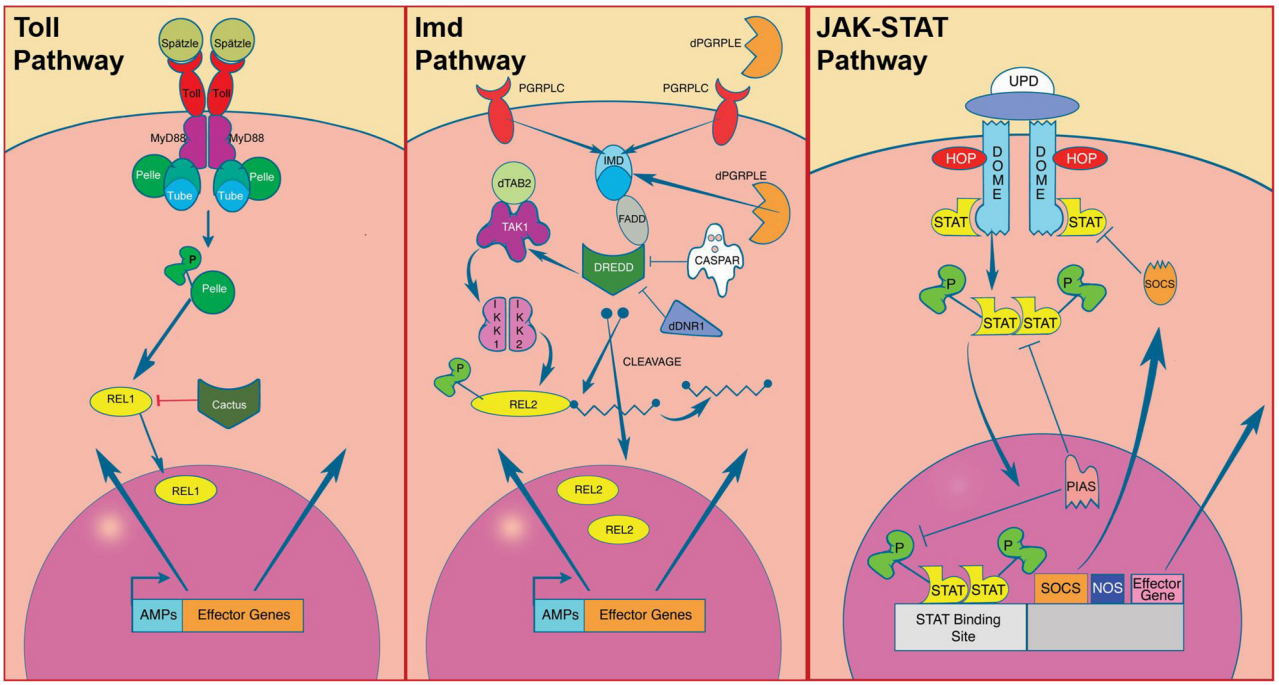

Fig. 1. The Toll, Imd and JAK-STAT Immune Signaling Pathways

Upon the recognition of bacteria or *Plasmodium*, the Toll pathway is stimulated by the 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, upregulating the transcription of immune genes that are responsible for microbial killing. Numerous studies conducted in *Anopheles* have highlighted the Imd pathway as the most efficient immune pathway in the defense against the human malaria parasite, *Plasmodium falciparum*. The Imd pathway is stimulated when the transmembrane PGRP-LC receptor recognizes bacteria or *Plasmodium*. This leads to a signaling cascade that will result in the cleavage of Rel2-F and the translocation of active Rel2-S into the nucleus, upregulating the transcription of immune genes. The JAK-STAT Immune Signaling Pathway has been implicated in antibacterial, antiviral, and antiplasmodial defense in mosquitoes. The JAK-STAT pathway is initiated by the binding of the cytokine ligand Unpaired (UPD) to the transmembrane receptor DOME. This then leads to the eventual nuclear translocation of STAT and transcriptional activation of immune effector genes.