CHAPTER 12

MOSQUITO IMMUNITY

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Abstract: Throughout their lifetime, mosquitoes are exposed to pathogens during feeding, through breaks in their cuticle and following pathogen-driven cuticular degradation. To resist infection, mosquitoes mount innate cellular and humoral immune responses that are elicited within minutes of exposure and can lead to pathogen death via three broadly defined mechanisms: lysis, melanization and hemocyte-mediated phagocytosis. This chapter reviews our current understanding of the mosquito immune system, with an emphasis on the physical barriers that prevent pathogens from entering the body, the organs and tissues that regulate immune responses and the mechanistic and molecular bases of immunity.

INTRODUCTION

Mosquitoes (Diptera: Culicidae), like all organisms, are under constant threat of infection. For the continuation of their life cycles, females of all anautogenous species are required to take a blood meal for the production of eggs. This act of blood feeding often exposes mosquitoes to blood-borne pathogens that aim to undergo complex developmental, reproductive and/or migrational processes inside a mosquito host before they can be transmitted during a subsequent blood meal. In addition to risking infection through blood feeding, mosquitoes often acquire pathogens through sugar feeding, through breaks in their cuticle that are created after physical injury and following pathogen-driven cuticular degradation. Whereas pathogen acquisition through blood feeding occurs exclusively during the adult life stage, infection through the cuticle is likely most prevalent during the aquatic developmental stages, when mosquitoes live in environments rife with bacteria.

Although culicine and anopheline mosquitoes are effective vectors of human and animal pathogens, susceptibility or resistance to infection is often the result of intricate co-evolutionary

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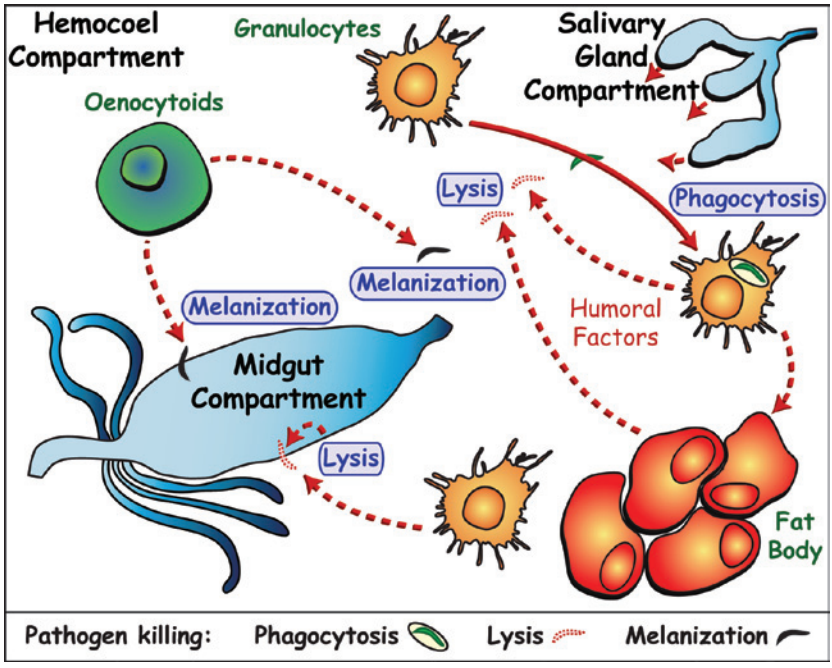

Figure 1. Mosquito immune responses in the three major immune compartments. In the hemocoel, granulocyte and oenocytoid hemocytes, as well as fat body, kill pathogens via phagocytosis, lysis and melanization. In the midgut, immune factors produced by epithelial cells, hemocytes and possibly fat body, kill pathogens via lytic and melanization pathways. Little is know about the role salivary glands play in immune responses, but they produce immune factors in response to infection.

processes in which mosquitoes and pathogens engage in counter-adaptations for survival and infection. As a consequence of this evolutionary arms race, only mosquitoes of the genus *Aedes* are capable of transmitting dengue fever virus and only *Culex* mosquitoes transmit Japanese encephalitis virus.[1] Similarly, of the greater than 3,000 known species of mosquitoes, only a subset of species from the genus *Anopheles* is capable of transmitting human malaria.[2] Even within the susceptible *Anopheles gambiae* species, some individuals are resistant to infection and others, while unable to eliminate the infection, are capable of drastically reducing pathogen numbers.[3,4] The specificity of mosquito-pathogen associations also varies among species of parasites. For example, the mosquito *Armigeres subalbatus* effectively transmits the filarial nematode *Brugia pahangi* but is resistant to a close relative, *Brugia malayi*.[5] Several factors account for the ability of pathogens to survive inside mosquitoes, including behavior (e.g., will the mosquito encounter the pathogen?) and physiological compatibility (e.g., are the correct conditions present in the host that allow the pathogen to complete its life cycle?). Another major factor that determines whether a pathogen can survive inside mosquitoes rests on the strong innate immune responses mounted by the host and on whether the pathogens have evolved mechanisms to evade these defenses.

In broad terms, pathogen killing by mosquitoes is accomplished by three primary mechanisms: cell-mediated phagocytosis, melanization and lysis (Fig. 1). Each is initiated by pattern recognition receptors and the factors leading to killing can be subdivided into cellular and humoral components (Fig. 2). The cellular response includes phagocytosis

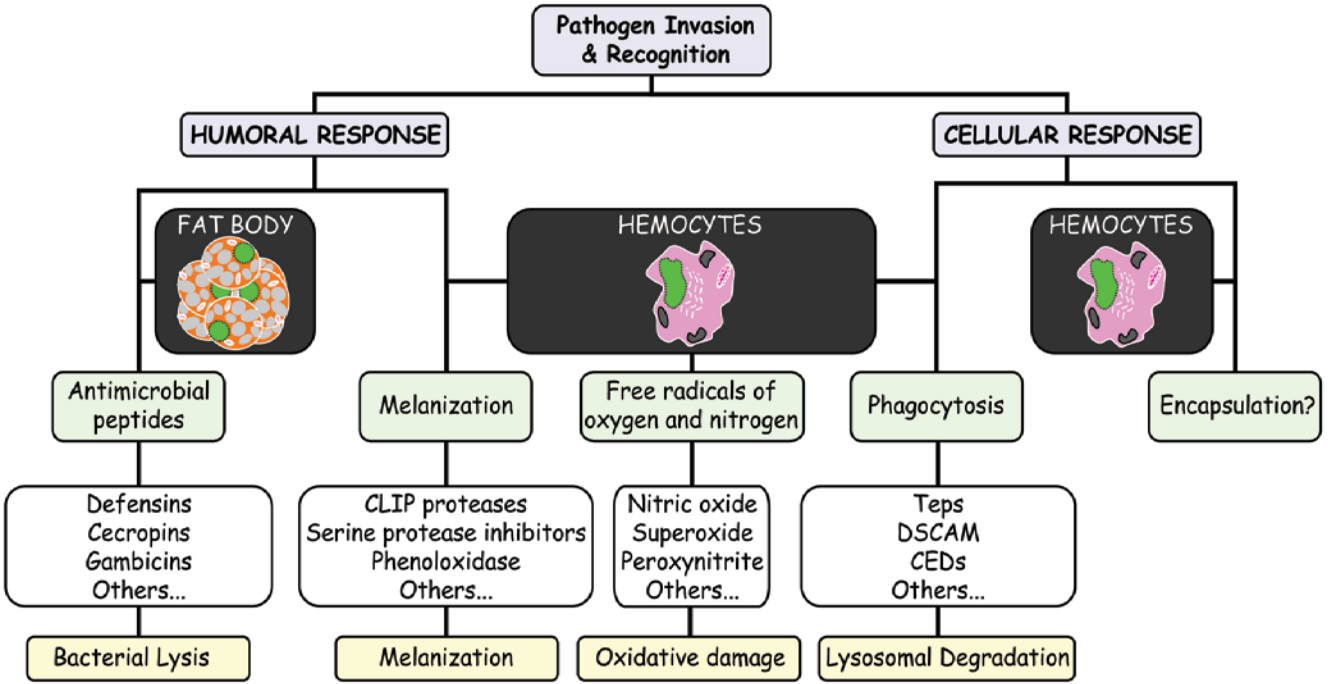

Figure 2. Immune responses in the mosquito hemocoel. Antimicrobial responses in the hemocoel are mediated by hemocytes and fat body and include antimicrobial peptides, melanization, reactive oxygen species, reactive nitrogen species and phagocytosis.

and encapsulation by hemocytes and pericardial cells.[6-9] The humoral response includes pattern recognition receptors, inducible antimicrobial peptides, the phenoloxidase cascade system of melanization and wound healing, and reactive oxygen and reactive nitrogen intermediates.[10-15] Regardless of this conceptual organization, the line between cellular and humoral immunity is blurred because many humoral components are produced by hemocytes and participate in cellular immune responses.[16,17] This chapter reviews the interactions between mosquitoes and pathogens, with emphasis placed on the physical barriers that prevent pathogens from entering the body, the organs and tissues that regulate immune responses, the mechanistic manifestations of immunity and our current understanding of the molecular basis of immunity.

BIOLOGY OF PATHOGENS INSIDE MOSQUITOES

Mosquitoes are subject to infection by viral, bacterial, fungal, protozoan and metazoan pathogens and initial entry into the host generally occurs either through breaks in the cuticle or by ingestion. The biology of pathogens inside the mosquito is dependent on their mode of transmission. By and large, bacteria and fungi enter mosquitoes through wounds in their outer cuticle or through the midgut epithelium after feeding. They quickly replicate in the host's gut or hemocoel (body cavity) and can be transmitted to a subsequent host while the initial host is alive or after its death. The time between colonization and transmission can be very short; some of these infectious agents are highly pathogenic and are lethal within hours of infection.[18] For that reason, bacterial and fungal pathogens are currently being used in the development of novel pest control strategies.[19,20]

Pathogens acquired and transmitted through blood feeding, on the other hand, must undergo obligatory processes that require their interaction with multiple tissue types and require that the host survives for days or weeks before transmission can take place.[21] *Plasmodium* parasites and arboviruses, for example, must cross the midgut epithelium, replicate, migrate through the hemocoel and invade the salivary glands before the mosquito can infect a subsequent host during her next blood feeding. Similarly, filarial nematodes must leave the midgut, develop in the thoracic musculature or Malpighian tubules and migrate to the mouthparts for the mosquito to become infectious. Unlike fungal and bacterial pathogens, the transmission of blood-borne pathogens requires mosquito viability throughout the entirety of the pathogen's life cycle inside the insect host. Nevertheless, these pathogens decrease fitness, reduce fecundity and, if acquired in large enough numbers, can be lethal to the mosquito.[22-25]

MOSQUITO COMPARTMENTS AND BARRIERS TO INFECTION

Pathogens inhabit three primary compartments in the mosquito: the midgut, the hemocoel and the salivary glands (Fig. 1). All three of these compartments include physical and physiological barriers that limit or reduce pathogen development. In addition, cells in all of these compartments produce immune factors with antimicrobial activity. These responses, though powerful, are innate and lack the properties of somatic hypermutation that are hallmarks of vertebrate adaptive immunity.[26]

The Gut Compartment

When pathogens enter mosquitoes via ingestion, the initial barrier faced is physical destruction by the cibarial armature.[27] This barrier, composed of sclerotized teeth and spines that protrude into the lumen of the foregut, slices large pathogens during the initial stages of ingestion and before they reach the midgut. The cibarial armature is effective in limiting infection by large metazoan parasites such as filarial nematodes but does little to destroy protozoan, bacterial and viral pathogens. Once in the midgut lumen, pathogens must survive digestive enzymes and invade the midgut epithelium by either digesting a thick acellular chitinaceous peritrophic matrix formed in response to blood feeding or by initiating epithelium invasion prior to the formation of this matrix.[28-30] The mechanisms leading to midgut penetration are not well understood, but depending on the pathogen may involve receptor-ligand interactions, physical burrowing through the epithelium and/or digestion of host cells.[31-33]

Mosquitoes drastically limit pathogen development in the midgut. For *Plasmodium* parasites, the causative agents of malaria, ookinete development in the gut results in a 500 to 100,000-fold reduction in parasites numbers and the ookinete to oocyst transformation that occurs on the basal side of the midgut experiences parasite losses of 5 to 100-fold.[34] The bases for these parasite reductions are complex, but include lytic and melanization events that are controlled by pattern recognition receptors, serine proteases and their inhibitors, and enzymatic cascades.[35-40] Indeed, molecular and biochemical studies have shown that the midgut rapidly produces a vast milieu of antimicrobial proteins in response to pathogen exposure.[41-45] In addition to these midgut-produced immune factors, infection of this organ triggers the production of immune proteins in other tissues, some of which are transported into the midgut where they exert their antimicrobial activities.[36,38,46,47]

The Hemocoel Compartment

The hemocoel compartment is an open body cavity that contains all visceral organs and is delineated by the outer cuticle and the basal lamina surrounding internal tissues. For many reasons, including preventing infection, insects have developed a tightly-sealed hydrophobic outer cuticle that shields internal organs from the outside environment. Breaks in this cuticle form temporary openings through which pathogens may enter. While these wounds commonly occur in nature, coagulation and melanization responses involving wound contraction, hemocyte degranulation and scar formation rapidly close these lesions.[48,49] As described above, pathogens also enter the hemocoel through ingestion followed by midgut penetration. Regardless of the mode of entry, pathogens disseminate throughout the hemocoel by either pathogen-driven active motility or the natural flow of hemolymph.[50-53]

Once in the hemocoel, pathogens are immersed in a nutrient-rich medium that contains immune cells and humoral immune factors produced by hemocytes, pericardial cells and fat body. Hemocytes are immunosurveillance cells that initiate innate immune responses and are found circulating with the hemolymph or attached to visceral tissues (Fig. 3). They are involved in the killing and sequestration of pathogens via phagocytosis, nodulation and the secretion of humoral immune factors.[6-9,12,54] Transcriptomic analyses in several mosquito genera have repeatedly shown the broad range of immune factors produced by hemocytes, which include pattern recognition receptors, proteins involved in phagocytosis, melanization modulators and enzymes, signal transduction proteins, stress response proteins

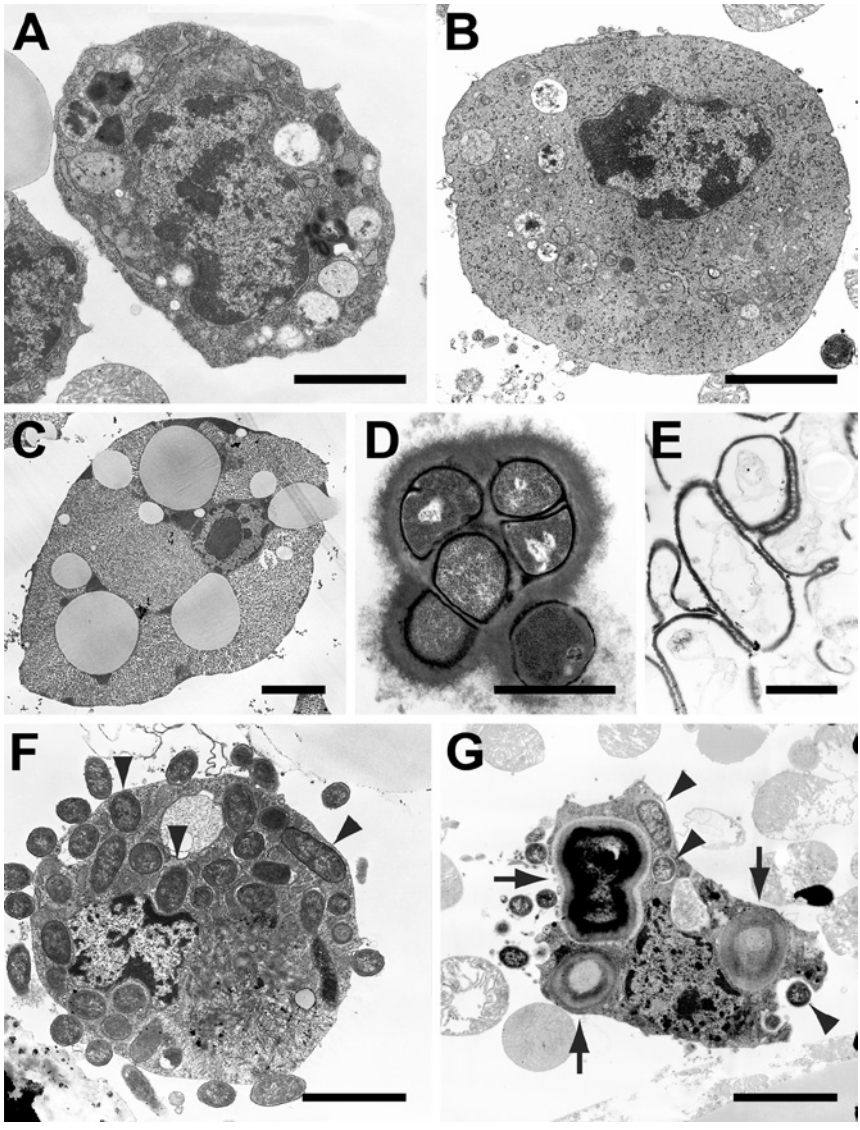

Figure 3. Transmission electron micrographs of immune cells and immune responses in the mosquito hemocoel. A) Circulating granulocyte. B) Circulating oenocytoid. C) Fat body. D) Melanization of *Staphylococcus aureus* in the hemocoel. E) Lysis of *S. aureus* in the hemocoel. F) Phagocytosis of *Escherichia coli* (e.g., arrowheads) by a circulating granulocyte. G) Phagocytosis of unmelanized *E. coli* (e.g., arrowheads) and melanized *Micrococcus luteus* (e.g., arrows) by a circulating granulocyte. Scale bars: A-B, 2 μm; C, 5 μm; D-E, 1 μm; F-G, 3 μm.

and antimicrobial peptides.[16,17,47,55] RNA interference-based transcriptional knockdown of several hemocyte-produced immunity genes results in increased susceptibility to infection with bacteria and malaria parasites, indicating that hemocytes and the effector molecules they produce are essential for efficient immune responses.[36,38,46,47,56]

On the basis of morphology, lectin binding properties and enzymatic activity, mosquito hemocytes were initially subdivided into granulocytes, oenocytoids, adipohemocytes and thrombocytoids.[57] The latter two subtypes were later determined not to be true circulating hemocytes but instead fat body and pericardial cells also collected during the extraction process.[9] A subsequent study confirmed the initial hemocyte classification system and postulated that a third cell type, progenitor prohemocytes, also circulates with the hemolymph.[6]

The number of circulating hemocytes in an adult mosquito is limited and mitotic activity has not been observed in these cells, suggesting that after eclosion no new hemocytes are produced. Adult mosquitoes contain slightly more than 1,000 circulating hemocytes at the time of emergence and the number of circulating cells drops with age, falling to 800 or less by their sixth day after eclosion.[6,58] Granulocytes account for approximately 95% of the circulating hemocyte population. They contain membrane-delimited vesicles, exhibit acid phosphatase activity and strongly adhere to artificial substrates following extraction from the mosquito (Fig. 3A).[8,57] Granulocytes also produce proteins involved in humoral immune pathways, such as nitric oxide synthase, serine proteases and serine protease inhibitors.[6,12] However, the most striking characteristic of granulocytes is their phagocytic capacity: granulocytes engage in the phagocytosis of bacterial pathogens within 5 minutes after exposure and as infections progress individual hemocytes dramatically grow in size to accommodate the internalization of hundreds of foreign entities (Fig. 3F-G).[9,12,54,58] Granulocytes have also been observed to phagocytose *Plasmodium* sporozoites, but the rate of sporozoite phagocytosis is low when compared to bacteria and the importance of this immune process in limiting *Plasmodium* infection remains unclear.[8,59]

The other major hemocyte class is the oenocytoid (Fig. 3B). These cells account for approximately 5% of circulating hemocytes and produce phenoloxidase and phenylalanine hydroxylase,[9,57,60] which are rate-limiting enzymes in the humoral melanization pathway.[61,62] These enzymes are present in the cytosol of oenocytoids and their sequences do not contain classical signal peptides,[60,63] indicating that enzyme release into the hemolymph must occur by either nonclassical secretion mechanisms or cell rupture. Although this process has not been resolved in mosquitoes, phenoloxidase release in lepidopterans and brachyceran dipterans has been shown to occur by cell rupture in an eicosanoid dependent manner.[64,65]

While mosquitoes mount strong and rapid immune responses following pathogen exposure, the type of immune response can vary depending on the pathogen. For example, the primary immune mechanism against *Escherichia coli* is phagocytosis, but against *Micrococcus luteus* is melanization.[8,9] Studies on the immune response mounted against a large panel of bacteria determined that the strength of phagocytosis versus melanization responses is not dependent on Gram-type, but the variable response illustrates that mosquitoes discriminate between pathogens.[54]

Additional evidence supporting the importance of hemocytes in immunity comes from a study showing that there is age-associated mortality in *Aedes aegypti* following *E. coli* immune challenge.[58] This mortality correlates with a decrease in the number of circulating hemocytes and a decrease in the ability to kill *E. coli*, but age has no effect on the transcription of the antimicrobial peptides cecropin, defensin, or gambicin.[58] These findings suggest that the increase in susceptibility is not due to antimicrobial peptide production but instead to a decrease in the number of circulating hemocytes available to quell the infection and are in agreement with a recent report in *D. melanogaster* showing that targeted ablation of hemocytes renders flies incapable of surviving bacterial infections.[66]

In addition to hemocytes, pericardial cells have been relentlessly implicated in immune surveillance, but no direct evidence has been published. Pericardial cells are large binucleate cells that flank the mosquito heart. In *Anopheles*, these cells have been reported to vary in number from 56 to greater than 300, depending on the observer, and also to uptake ammonia carmine dye.[67,68] More recent studies have suggested that pericardial cells contain several immune-related molecules,[69,70] but whether these cells actually produce these proteins or sequester them from the hemolymph is not known. In addition, intense phagocytic activity has been reported near the surface of the mosquito heart, an area densely populated by pericardial cells. However, it remains unclear whether this phagocytic activity is carried out by pericardial cells, sessile hemocytes, or yet unidentified immune cells.[59] Nevertheless, the location of effector cells in the vicinity of the heart is advantageous for immune surveillance and pathogen destruction, as their position in areas of high hemolymph flow increases their probability of encountering invading pathogens.[51]

While hemocytes and possibly pericardial cells mediate both cellular and humoral responses, the fat body's role in immunity is exclusively humoral. The fat body is a multifunctional organ consisting of loosely assembled cells that are rich in glycogen and lipids and line the mosquito integument (Fig. 3C).[57,71] Among its many functions, fat body synthesizes vitellogenin precursors required for the production of eggs, serves in energy storage and produces numerous hemolymph components.[72] Specifically, immune activity in the fat body includes infection-induced production of antimicrobial peptides, reactive oxygen species and reactive nitrogen species.[12,15,73-75]

The Salivary Gland Compartment

Transmission of many viral and protozoan parasites to a vertebrate host requires their injection with the mosquito saliva during blood feeding. Hence, invasion of the salivary gland epithelium and migration into the salivary duct is a requirement for the continuation of the life cycle of these pathogens. The salivary gland epithelium forms a physical barrier that pathogens must cross and *Plasmodium* parasites as well as other pathogens have evolved proteins that drive invasion by first binding to specific mosquito salivary gland surface factors.[76-78] To date, little is known about the role of the salivary glands in antimicrobial responses, but several gene expression studies have described the production of immune proteins in the salivary glands of naïve mosquitoes, *Plasmodium*-infected mosquitoes and mosquitoes that have ingested a noninfectious blood meal.[79-86] Empirical evidence showing that the salivary glands serve as an active immune organ is largely lacking, with the exception of a single publication showing that a serine protease inhibitor (SRPN6) produced in the salivary epithelium limits gland invasion by *Plasmodium* sporozoites.[87]

MOLECULAR BASIS OF MOSQUITO IMMUNITY

The publication of the *An. gambiae* and *Ae. aegypti* genomes has led to an explosion in the number of studies focusing on mosquito immunity.[88-91] Many of these studies have employed homology searches to identify putative immune genes and infer their function. These bioinformatic observations, together with gene expression data and transcriptional manipulations using RNA interference or transgenesis, have allowed researchers to conclusively identify genes that are required for pathogen suppression.

Conversely, similar approaches have been used to identify mosquito genes that facilitate pathogen survival.

Pattern Recognition Receptors

Invading pathogens are recognized by the molecular interaction between host-derived pattern recognition receptors (PRRs) and pathogen associated molecular patterns (PAMPs). Bioinformatic analysis of the *An. gambiae* genome has identified approximately 150 putative PRRs.[92] Most are secreted proteins that contain adhesive domains capable of interacting with PAMPs and cluster as members of large gene families. While experimental evidence has shown that many are involved in immune responses, their actual role as PRRs has not been cemented, as their recognized PAMPs have not been identified.

Thioester containing proteins (TEPs) are hemolymph proteins involved in the killing of bacteria and *Plasmodium* ookinetes.[41,46,93,94] Members of the TEP gene family share structural similarities with α2-macroglobulins and vertebrate complement components C3, C4 and C5. By and large, most studies on mosquito TEPs have focused on the hemocyte-produced phagocytosis enhancer TEP1. This protein is secreted into the hemocoel as a single chain molecule that is activated by proteolytic cleavage.[93] Cleaved TEP1 is then stabilized by forming a complex with the leucine rich repeat containing proteins LRIM1 and APL1C prior to binding bacteria in the hemocoel or ookinetes in the midgut, triggering their destruction.[36,38,93] The antiplasmodial activity of APL1 and TEP1 are further supported by studies showing that genetic variation at their respective loci has a profound effect on immune competence against *Plasmodium*.[4,95] A recent genome-wide mapping of reciprocal crosses of mosquito strains that are susceptible or resistant to *P. berghei* demonstrated that polymorphisms in Tep1 explain a portion of the variability in *P. berghei* killing efficiency observed among laboratory mosquito colonies.[95] While this study was published years after the discovery of Tep1, APL1 was initially characterized because it is coded within a *Plasmodium*-resistance island that explains naturally occurring resistance to *P. falciparum* in field-caught *An. gambiae*.[4] Dissection of the APL1 locus revealed that it is composed of three genes, all of which display major structural haplotypes.[96] Interestingly, APL1C is solely responsible for resistance to *P. berghei*, but distinct haplotypes in the neighboring APL1A gene are associated with various levels of resistance.[96]

C-type lectins are soluble or membrane bound proteins that bind carbohydrates in a calcium-dependent manner. In *An. gambiae*, C-type lectins are both positive and negative regulators of mosquito immune responses. In the midgut, CTL4 and CTLMA2 function as negative regulators of the melanization of *Plasmodium berghei* ookinetes.[37] However, in the hemocoel these same C-type lectins are present in the hemolymph as disulfide-linked heterodimers that function in the killing of *E. coli* in a melanization-independent manner.[97] Transcriptional knockdown of either of these lectins increases bacterial proliferation in the hemocoel and decreases mosquito survival, indicating that they are essential players in the antibacterial response.

Gram-negative binding proteins (GNBPs) were initially identified in *An. gambiae* because they share sequence similarities with GNBPs of other insects and because they are transcriptionally upregulated following infection with bacteria and *Plasmodium* parasites.[98] Six members of this gene family are expressed in *An. gambiae* and all presumably function as PRRs by binding ß-1,3-glucan and lipopolysaccharide on the surface of pathogens. GNBPs are transcribed in multiple tissues (hemocytes, midgut, salivary glands) and while

they are all upregulated following an immune challenge, they vary in their antimicrobial specificities. GNBP4, for example, participates in the killing of *E. coli*, *Staphylococcus aureus* and *P. berghei*, but not *Plasmodium falciparum*. In contrast, GNBPA2 participates in the killing of *E. coli* and *P. falciparum*, exhibits mild activity against *P. berghei* and is ineffective against *S. aureus*.[99]

The immunoglobulin superfamily consists of 138 genes in *An. gambiae*, 85 of which are upregulated following an immune challenge. Six of these genes were recently extensively characterized: two are involved in the killing of *P. falciparum* ookinetes (IRID4 and 6), two control the growth of opportunistic bacteria (IRID3 and 4) and three are involved in the killing of exogenously introduced bacteria.[100] Another member of this gene family, AgDSCAM, has been shown to opsonize bacteria and to kill the midgut stages of *Plasmodium*.[101]

Fibrinogen-related proteins (FREPs) represent a PRR family that has experienced massive expansion in mosquitoes.[102] Fifty-nine and 37 FREPs have been identified in *An. gambiae* and *Ae. aegypti*, respectively, compared to 14 in *Drosophila melanogaster*.[91] Functional studies have shown that the majority of mosquito FREPs are upregulated following an immune challenge and that many are essential for the killing of bacteria and the maintenance of immune homeostasis.[11,103] Several also have antiplasmodial activity and one in particular (FBN9) binds ookinetes as they invade the midgut epithelium.[11]

Immune Signaling

Microorganism recognition by PRRs can lead to pathogen destruction through constitutive effector mechanisms and/or the activation of intracellular signaling pathways that activate the transcription of effector genes. The major immune signaling pathways in mosquitoes are Toll, Imd and JAK/STAT and the path that leads to activation of these pathways may be amplified or repressed by modulatory proteins such as serine proteases and serine protease inhibitors. Most of the components in these signaling and regulatory pathways are conserved among dipteran insects.[91]

In mosquitoes, genes regulated by the Toll pathway are controlled by the NF-κB transcription factor Rel1. This pathway is induced by fungi, Gram(+) bacteria, viruses and *Plasmodium*. Induction of the Toll pathway by silencing of the negative regulator of Rel1, Cactus, dramatically decreases *P. berghei* and *Plasmodium gallinaceum* infection intensity in the *Anopheles* and *Aedes* midgut, respectively.[104,105] Co-silencing Rel1 and Cactus renders mosquitoes susceptible to infection, indicating that Cactus-mediated susceptibility is due to repression of Rel1.[104] Co-silencing of Cactus and LRIM1 or Tep1 also renders mosquitoes susceptible to infection, suggesting that these two effector molecules are induced through the Toll pathway. In addition to the antiplasmodial activity of genes induced through Rel1, the Toll pathway is also involved in controlling infection against entomopathogenic fungi and dengue virus.[106,107]

The Imd pathway is controlled by the NF-κB transcription factor Rel2. Rel2 exists as short (Rel2S) and full-length (Rel2F) forms, both of which are involved in the immune response against bacteria and *Plasmodium*.[104,108] Interestingly, while both the Toll and Imd pathways are involved in immunity against *P. berghei*, immunity against *P. falciparum* is controlled primarily through the Imd pathway, as transcriptional knockdown of the Imd negative regulator Caspar and not the Toll negative regulator Cactus renders *An. gambiae* resistant to infection.[109]

The least studied immune pathway in mosquitoes is the JAK/STAT pathway. In *An. gambiae*, this pathway is controlled through two STAT transcription factors that are the result of a gene duplication event. In mosquitoes, activation of the STAT pathway requires STAT-B mediated activation of STAT-A, which is regulated by a negative feedback loop controlled by the signaling suppressor protein SOCS. Activation of this pathway leads to the induction of nitric oxide synthase transcription, which is a positive regulator of *Plasmodium* infection.[110] More recent studies have also implicated the JAK/STAT pathway in the immune response against dengue virus in *Ae. aegypti*. Here, inactivation of the JAK/STAT pathway by depletion of the receptor Domeless results in increased viral loads.[111] Conversely, hyperactivation of the pathway by depletion of the negative regulator PIAS results in a more resistant phenotype.

Antimicrobial Peptides

Antimicrobial peptides (AMPs) are secreted low molecular weight proteins that were initially identified for their antimicrobial activity in vitro.[74,75,112,113] Defensins, cecropins and gambicins comprise the three main AMP gene families in mosquitoes. In vitro analyses of their antimicrobial spectra showed that cecropins and gambicin are cytotoxic primarily against Gram(–) bacteria and defensins are cytotoxic primarily against Gram(+) bacteria.[74,75,112] Transcriptional regulation of AMPs occurs through the Toll and Imd pathways,[114,115] and all are transcriptionally upregulated in fat body following exposure to viruses, bacteria, *Plasmodium* and filarial nematodes.[16,107,116-118]

While defensins have antimicrobial activity in vitro, their function as essential components of the mosquito immune response continues to be debated. In *An. gambiae*, transcriptional knockdown of Defensin decreases mosquito survival following *S. aureus* infection,[119] but RNAi-based silencing of *Ae. aegypti* Defensin has no effect on mosquito survival following challenge with three bacterial species.[120,121] The role of cecropins in the antibacterial response in vivo is not known, but ectopic expression of a cecropin transgene results in increased killing of *P. berghei* ookinetes.[122] Lastly, Gambicin exhibits antiplasmodial activity in the midgut, as well as antibacterial activity in the hemocoel.[41]

Phenoloxidase-Based Melanization

Melanization in insects is essential for cuticle hardening, egg chorion tanning, wound healing and immunity. In mosquitoes, melanization (also known as melanotic encapsulation) is an immune effector mechanism involved in the killing of *Plasmodium*, filarial nematodes and bacteria and is visually manifested as a darkened proteinaceous capsule that surrounds invading pathogens (Fig. 3D).[5,8,35] Melanization involves a series of reactions that include the conversion of tyrosine to melanin precursors and the cross-linking of proteins to form a layer of melanin that surrounds and sequesters invading pathogens (Fig. 4).[10] Melanization often results in pathogen death but the killing mechanism remains unclear. It has been hypothesized that death may be caused by either oxidative damage brought on by unstable intermediates created during melanogenesis or by starvation, since the foreign agent becomes isolated from the nutrient-rich hemolymph.[123,124] Besides functioning as a killing mechanism, melanization is also involved in the clearing of already dead or dying pathogens.[37,40]

The process of melanization begins with the proteolytic cleavage of a pro-phenoloxidase zymogen into its active form. The exact sequence of events that leads

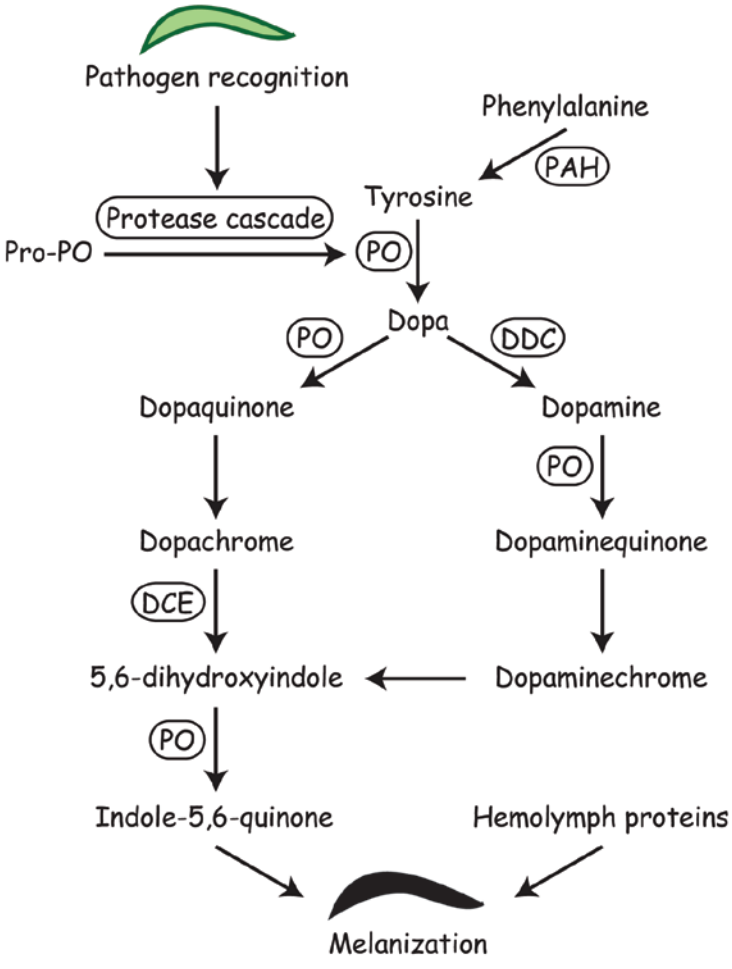

Figure 4. Proposed biochemical pathway leading to the melanization of pathogens. PAH, phenylalanine hydroxylase; PO, phenoloxidase; DDC, dopa decarboxylase; DCE, dopachrome conversion enzyme.

to this cleavage is not well understood, but involves the coordinated action of pattern recognition receptors, serine proteases and serine protease inhibitors. In the hemocoel, ß-1,3-glucan recognition protein is required for the melanization of filarial nematodes and bacteria by functioning as a pattern recognition receptor.[56,125] In the midgut, various proteins serve as promoters and inhibitors of melanization, with their exact role varying greatly between mosquito strains. For example, in *An. gambiae*, the *Plasmodium*-resistant L3-5 strain naturally melanizes *P. berghei* ookinetes but the G3 strain is susceptible to infection. Interestingly, mosquito C-type lectins function as repressors of melanization in the susceptible strain, as silencing CTL4 and CTLMA2 results in the LRIM1-dependent induction of ookinete melanization.[37] CLIP domain serine proteases also modulate the melanization response against *P. berghei* ookinetes and share structural similarities with pro-phenoloxidase activating enzymes from other insects.[126] However, once again, the

genetic background of the mosquito has a profound effect on the function of CLIPs.[40,126] For example, CLIPA7 inhibits melanization in the susceptible strain but has no effect on the resistant strain. Conversely, CLIPB3 has no effect on the susceptible strain but promotes melanization in the resistant strain. Similar observations on the role of CLIPs as inhibitors and promoters of melanization have been made in the mosquito hemocoel after intrathoracic injection of sephadex beads.[127]

In addition to CLIPs, mosquito serine protease inhibitors (SRPNs) modulate melanization responses.[128] Both SRPN6 and SRPN2 inhibit the melanization of *P. berghei* ookinetes in the resistant strain and SRPN2 also inhibits the spontaneous formation of melanin-based pseudotumors in the hemocoel.[129,130] These pseudotumors drastically reduce mosquito fitness, underscoring the importance of tightly regulating this immune process. Moreover, the factors that trigger melanization in the midgut are at least partially dependent on the host-parasite combination: SRPN2, CTLs and LRIM1, molecules that regulate development of *P. berghei* in the midgut, have no detectable effect on the development of the human malaria parasite *P. falciparum*.[131,132] These findings are not surprising given that *P. berghei* infection triggers the transcriptional regulation of over twice as many genes as *P. falciparum*.[41]

Once phenoloxidase becomes activated, the formation of melanin is initiated by the phenoloxidase-mediated hydroxylation of tyrosine to form dopa (Fig. 4).[133] Dopa is then oxidized by phenoloxidase to form dopaquinone, which is then converted to dopachrome. Dopachrome conversion enzyme converts dopachrome to 5,6-dihydroxyindole, which is oxidized into indole-5,6-quinone by phenoloxidase and then cross-linked with hemolymph proteins to form melanotic capsules. In an alternative pathway, dopa formed by the hydroxylation of tyrosine is decarboxylated by dopa decarboxylase to form dopamine, which is then converted into melanin by phenoloxidase and other enzymes. Empirical testing has shown that pathogen-induced melanin formation is accomplished via both of these pathways, as transcriptional knockdown of phenoloxidase, dopachrome conversion enzyme and dopa decarboxylase all lead to impaired melanization responses.[62,134-136] Throughout these reactions, tyrosine remains the rate-limiting substrate and endogenous production of tyrosine is accomplished by the hydroxylation of phenylalanine by phenylalanine hydroxylase. This latter reaction is essential for the melanization of filarial nematodes but not sephadex beads,[61,136] with the observed difference possibly related to the larger surface area that needs to be melanized following filarial worm infection. Finally, while melanization events are extracellular, many melanin-producing enzymes (e.g., phenoloxidase, dopachrome conversion enzyme, dopa decarboxylase and phenylalanine hydroxylase) are produced by circulating hemocytes,[9,57,60,134,135] and melanized pathogens are often subsequently phagocytosed by granulocytes (Fig. 3G).[8,9,54]

Nitric Oxide and Other Reactive Species

Nitric oxide is a multifunctional free radical created during the oxidation of L-Arginine to L-Citrulline by the enzyme nitric oxide synthase.[137] In *Anopheles*, nitric oxide synthase is a single copy gene with 18-22 distinct transcripts. Three of these transcripts are induced by *Plasmodium* infection,[138,139] and at least one is induced by bacterial infection.[12] In the midgut of the mosquito, *Plasmodium* glycosylphosphatidylinositols and *Plasmodium*-derived hemozoin acquired with an infectious blood meal induce the transcription of nitric oxide synthase through the STAT pathway,[43,110,140] and the resultant

nitric oxide kills *Plasmodium* ookinetes via lysis.[14,141,142] In the hemocoel, nitric oxide synthase is transcriptionally upregulated following bacterial infection and the production of nitric oxide is required for bacterial killing and mosquito survival during systemic infections with *E. coli*.[12]

Reactive oxygen species (ROS) kill *Plasmodium* ookinetes in the midgut and bacteria in the hemocoel.[15,143,144] While the exact mechanism of action against bacteria remains unknown, ROS kill *Plasmodium* through both lytic and melanization pathways.[15,143,144] The *P. berghei*-resistant L3-5 strain of *An. gambiae* lives in a constant state of oxidative stress that promotes melanization of ookinetes as they traverse the midgut epithelium.[143] Conversely, the susceptible G3 strain kills ookinetes via a lytic mechanism that is dependent on infection-induced oxidative stress that is maintained by the repression of catalase, an enzyme that breaks down hydrogen peroxide into oxygen and water.[15] Furthermore, reactive oxygen and reactive nitrogen species are intimately linked in mosquito immunity: peroxidases in the mosquito midgut use nitrite and hydrogen peroxide to synthesize highly reactive nitrogen dioxide and hydrogen peroxide triggers the transcriptional induction of nitric oxide synthase.[145,146]

Phagocytosis

Phagocytosis is an evolutionarily conserved immune process used for the killing and sequestration of small microorganisms. In this process, a particle is recognized, bound by proteins in the plasma membrane and internalized into a membrane-delimited phagosome. The phagosome then fuses with a lysosome and hydrolytic enzymes digest the particle. In mosquitoes, the granulocyte subpopulation of hemocytes uses this immune process to sequester and kill bacteria as early as 5 minutes after exposure.[8,9] Approximately 95% of circulating hemocytes are phagocytic and it has been estimated that individual hemocytes are capable of phagocytosing over 1,000 bacteria within 24 hours of infection.[58]

Several studies have investigated the molecular basis of phagocytosis by visualizing the uptake of fluorescently labeled dead bacteria by mosquito immortal cell lines or by low magnification fluorescence microscopy of whole mosquitoes.[93,94,147] Identified regulators of phagocytosis include pattern recognition receptors, transmembrane receptors and intracellular signaling proteins.

Thioester containing proteins TEP1, TEP3 and TEP4 are involved in the phagocytosis of Gram(+) and Gram(–) bacteria in the mosquito hemocoel.[93,94] The modes of action of TEP3 and TEP4 are not known, but proteolytically activated TEP1 opsonises bacteria by thioester-mediated binding, which in turn initiates phagocytosis. In addition to the above TEPs, the leucine rich repeat containing protein LRIM1 is also required for phagocytosis.[94] The exact mechanism by which LRIM1 functions has not been resolved, but TEP1-based antimicrobial activity in the midgut is dependent on the complexing of TEP1 with LRIM1 in the hemocoel.[36]

AgDSCAM is a hypervariable immunoglobulin that is encoded by 101 exons, which can be transpliced into over 31,000 variants.[101] AgDSCAM binds bacteria in vitro and triggers their phagocytosis by an immortal hemocyte-like cell line. In vivo transcriptional knockdown of AgDSCAM increases bacterial proliferation in the hemocoel and decreases mosquito survival, suggesting that AgDSCAM mediated phagocytosis or humoral killing is required for effective antibacterial responses in the hemocoel.

Several transmembrane receptors have also been implicated in phagocytosis and may function by either directly recognizing pathogens or by recognizing pathogens that

have been opsonized by hemolymph proteins. Among these are a ß integrin (BINT2), a peptidoglycan recognition protein (PGRPLC) and a low-density lipoprotein receptor-related protein (LRP1).[94,147]

Lastly, several intracellular proteins trigger the internalization of bacteria. Transcriptional knockdown of *An. gambiae* CED2, CED5, or CED6 reduces phagocytosis efficacy by up to 80%.[94] Epistatic analyses then showed that TEP1, TEP3, LRIM1 and LRP1-mediated phagocytosis occurs through the CED6 pathway and TEP4 and BINT2-mediated phagocytosis occurs through the CED2/CED5 pathway. The involvement of mosquito CEDs in the phagocytosis of foreign bodies through two genetically independent pathways is significant but not unexpected. Cell death abnormal genes were initially discovered in *Caenorhabditis elegans* during screens aimed at identifying genetic factors required for the phagocytosis of apoptotic bodies in the developing worm and these factors function through two independent but partially redundant pathways: the CED-1/CED-6/CED-7 pathway and the CED-2/CED-5/CED-10/CED-12 pathway.[148-150]

CONCLUSION

The field of mosquito immunity has experienced unprecedented growth in the past decade. The publication of the *An. gambiae* and *Ae. aegypti* genomic sequences has led to the bioinformatic identification of numerous putative immunity genes.[88-91] Technical advances such as RNA interference, paratransgenesis and transgenesis have then allowed researchers to empirically test their function in effecting or regulating immune responses against diverse groups of pathogens.[119,151,152] As a result of these studies, it has become apparent that the mosquito immune system shares numerous similarities with vertebrate immune systems. Immune responses in both vertebrates and invertebrates are initiated by microbe recognition events that trigger signaling pathways and effector mechanisms. Moreover, similar to vertebrates, mosquitoes recognize pathogens using complement-like cascades, possess phagocytic cells that circulate with the blood and transcribe effector molecules through Toll and JAK/STAT pathways. Further studies on mosquito immunity will continue to shed light on the evolution of these complex and essential responses.

In addition to the evolutionary conservation of immune components, obtaining a better understanding of the mosquito immune system may translate into novel public health interventions. Mosquitoes are cosmopolitan pests and disease vectors.[153] Because of their global importance, these organisms are subjects of constant study in efforts to uncover mechanisms that reduce their population densities as well as their ability to transmit disease. One aspect of mosquito biology that continues to receive considerable attention is their ability to fight infectious agents, as it has been hypothesized that understanding how mosquitoes kill microbial pathogens may allow us to exploit weaknesses that increase the population-reduction effectiveness of biological control strategies, or to strengthen immune responses such that otherwise susceptible mosquitoes are rendered resistant to infection, halting disease transmission cycles.[19,20,154,155] Much must be done before the feasibility of these approaches can be further considered, including the continued expansion of work in immunity to wild mosquito populations and to a broader range of mosquito species in order to ensure that our current understanding of mosquito immunity is representative of natural host-pathogen interactions.

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