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Arthropod Innate Immune Systems and Vector-Borne Diseases

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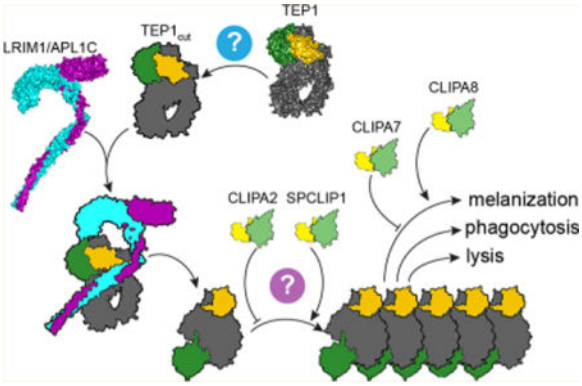
Abstract

Arthropods, especially ticks and mosquitoes, are the vectors for a number of parasitic and viral human diseases, including malaria, sleeping sickness, Dengue, and Zika, yet arthropods show tremendous individual variation in their capacity to transmit disease. A key factor in this capacity is the group of genetically encoded immune factors that counteract infection by the pathogen. Arthropod-specific pattern recognition receptors and protease cascades detect and respond to infection. Proteins such as antimicrobial peptides, thioester-containing proteins, and transglutaminases effect responses such as lysis, phagocytosis, melanization, and agglutination. Effector responses are initiated by damage signals such as reactive oxygen species signaling from epithelial cells and recognized by cell surface receptors on hemocytes. Antiviral immunity is primarily mediated by siRNA pathways but coupled with interferon-like signaling, antimicrobial peptides, and thioester-containing proteins. Molecular mechanisms of immunity are closely linked to related traits of longevity and fertility, and arthropods have the capacity for innate immunological memory. Advances in understanding vector immunity can be leveraged to develop novel control strategies for reducing the rate of transmission of both ancient and emerging threats to global health.

Abstract



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Notes

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The phylum arthropoda—crustaceans, arachnids, and insects—is by far the largest in the animal kingdom. Insects play key roles in natural ecosystems, pollinate our crops, make honey, and can even be food! Yet blood-feeding, or hematophagous, arthropods—mosquitoes, sand flies, ticks, etc.—serve as vectors for devastating human diseases, including malaria, sleeping sickness and Chagas disease, leishmaniasis, Dengue, and Zika virus (Table 1). For vector-borne diseases, transmission represents a vulnerable and attractive point of attack.[1] Vector control remains the most generally effective measure for preventing malaria transmission[2] and is essential for the control of Dengue and Zika outbreaks in the absence of safe and effective vaccines.

A notable feature of arthropod disease vectors is the tremendous variation observed between individuals in their susceptibility to infection by the pathogen (known as vectoral capacity). *Plasmodium* (the causative agent of malaria) infects *Anopheles* mosquitoes, while Dengue virus infects *Aedes* mosquitoes, but not vice versa. A major part of this variation is due to genetically encoded factors such as immunity. In agriculture, beneficial insects such as honey bees are threatened by numerous pathogens, including viruses, bacteria, fungi, protozoa such as *Nosema*, and mites such as *Varroa*. Simultaneously, viruses (e.g., nuclear polyhedrosis virus), bacteria (e.g., *Bacillus thuringiensis*), fungi (e.g., *Beauveria bassiana*), and nematodes (e.g., *Heterorhabditis*) are used for biological control of agricultural and nuisance pests. For all these reasons, a range of researchers have become increasingly interested in the immune response of insects.

Arthropods rely solely on innate immunity to protect themsleves from infectious agents. The study of innate immunity in insects has led to major advances in human immunology, such as the discovery of Toll-like receptors.[3] Arthropod physiology is simpler than that of vertebrates, with a single internal body cavity (hemocoel) and circulating fluid (hemolymph), but the principal components of innate immunity are conserved between the two phyla. These include (i) physical and chemical barriers to infection at epithelial surfaces, (ii) circulating proteins that detect, bind, or neutralize pathogens (humoral responses) by processes such as lysis, agglutination, and melanization, and (iii) circulating cells (hemocytes) that combat pathogens by processes of phagocytosis and encapsulation.[4]

These components communicate in a structured response to infection. First, pattern recognition receptors (PRRs) detect pathogen-associated molecular patterns (PAMPs) or “danger signals” associated with cell damage or stress.[5,6] Second, PRRs activate effector proteins in the hemolymph (extracellular pathogen) or cytoplasm (intracellular pathogen) and signal transduction in surrounding tissues via the Toll, Imd, JNK, and JAK/STAT pathways, resulting in multiple transcriptional responses. Third, pathogen-bound PRRs and effector proteins recruit immune cells to phagocytose or encapsulate pathogens, while transcriptional responses ramp up secretion of antimicrobial peptides and other effector proteins to neutralize extracellular infections, RNAi, and other antiviral pathways to restrict viral infection and apoptotic/autophagic pathways to kill intracellular pathogens or the infected cell itself. Finally, wound healing and clearance pathways such as melanization restore the integrity of physiological barriers.

Recent structural and functional studies, leveraged by accompanying advances in genomics and proteomics, show that despite their similarities a vast font of biochemical diversity exists in the immune systems of arthropods compared to that of vertebrates. In the absence of adaptive immunity and under conditions of *r*-selection, a high degree of allelic variation in immune genes and repertoires of immune complexes provide robustness and adaptation of a population in the face of new pathogens. This review covers the molecular mechanisms of innate immunity in arthropods with a specific focus on insect and tick vectors of human disease. First, the physiology of insect immunity and routes of pathogen transmission are outlined. Second, the structure and function of molecules engaged in the principal components of arthropod immunity are presented, and their roles within the different components of the immune response and against distinct classes of pathogens. The review concludes with the current state of application of this knowledge in the control of arthropod populations and an outline of the many unanswered questions in the field.

PHYSIOLOGY, IMMUNITY, AND VECTOR-BORNE PATHOGENS

An overview of arthropod physiology and the life cycles of vector-borne pathogens is shown in Figure 1. An insect's first line of defense is an exoskeleton formed of chitin, a polymer of β-1,4-*N*-acetylglucosamine.[7] Linear polymers form antiparallel crystalline fibers of α-chitin. These nanofibers are embedded in a protein/lipoprotein matrix cross-linked by two related processes. Sclerotization is the modification and cross-linking of peptides by a range of *o*-diphenols derived from *N*-acetyl dopamine (NADA) and *N*-β-alanyl dopamine (NBAD), while melanization is the formation of phenolic polymers from tyrosine via the intermediate dopachrome.[8] Both processes are catalyzed by phenol oxidase (PO), a type 3 dicopper enzyme homologous to catechol oxidase and tyrosinase.[9,10] The cuticle is formed by hard, impermeable lamellae composites of sclerotized chitin. A porous, nonlamellae chitinous membrane, the peritrophic matrix (PM), is secreted from the epithelium of the gut to physically separate the contents of the lumen,[11] and chitin microfibrils run as spirals along the inside of the trachea.

Beneath these barriers lie epithelial cells. Midgut epithelial cells secrete proteins and glycans from their apical surface to form a mucosal extracellular matrix and a layer of carbohydrate known as a glycocalyx to insulate the plasma membrane from the lumen. Epithelial cells form tight junctions, known as septate junctions (SJ), to create an impermeable barrier between the exterior and the hemocoel and secrete immune sensing/effector proteins into the lumen and the hemocoel. Beneath the epithelium, a second physical barrier, the basal lamina, composed of collagen, laminin, and proteoglycans separates the epithelium from the circulatory fluid, or hemolymph.

The insect hemolymph volume and cell density vary greatly among insects (0.3–0.4 μL, 10^3–10^4 cells in *Drosophila, Anopheles*, and *Aedes*).[12] Hemolymph contains several types of cells, or hemocytes: (i) plasmatocytes and granulocytes, (ii) lamellocytes, and (iii) oenocytoids or crystal cells.[13] Phagocytosis, the engulfment of bacteria and other small particles, is performed by plasmatocytes (*Drosophila melanogaster*) or granulocytes (*Anopheles gambiae*). Some granulocytes facilitate agglutination, clumping pathogens and hemocytes together, or coagulation of hemolymph in response to wounding. Lamellocytes

encapsulate particles too large to be phagocytosed, forming an impervious barrier several cells thick. Oenocytoids release prophenol oxidase (PPO) that is converted to PO, catalyzing melanization of agglutinated/encapsulated particles and wound healing. Hemocytes secrete immune factors into the hemolymph to both sense and engage pathogens. Additional immune factors are secreted from the fat body, a mesodermal tissue distributed throughout the hemocoel.

Insects face constant assaults by pathogens through ingestion or inhalation. Pathogens follow a variety of life cycles within their insect vector. Baculoviruses, bacteria, fungi, and *Plasmodium* parasites secrete chitinases to penetrate chitinous barriers. A number of pathogens complete their life cycle within the digestive tract. Kinetoplastids escape from the posterior end of the PM, from which they migrate to the mouth (*T. brucei* and *Leishmania major*) or anus (*T. cruzi*) to exit the insect vector upon the next blood meal.[14,15] *Y. pestis* (plague) forms biofilms in fleas' mouth parts (proventriculus) that result in transmission to a host upon subsequent blood meals, while flea and louse-borne *Rickettsia* (typhus) are excreted in feces.[16]

Pathogens, including arboviruses such as Dengue and Zika, bacteria such as *Borrelia* and *Rickettsia*, and *Plasmodium* parasites, can also traverse the hemocoel to reach the mouth parts (horizontal transmission) or ovaries (vertical transmission). Viruses are intracellular pathogens, initially infecting the midgut epithelium, then secondary tissues such as hemocytes and the fat body, and eventually salivary glands and ovaries. The rickettsiae are intracellular pathogens that invade the hemocoel; some species are pathogenic to the insect vector. The rickettsiae, like arboviruses, are capable of transovarial transmission.

The extracellular bacterium *Borrelia* migrates from the midgut of a feeding tick to the salivary gland to infect a vertebrate host and is then reingested and infects the vector.[17] *Plasmodium* parasites are extracellular pathogens, traversing the midgut epithelium to form cysts between the basal labyrinth and the basal lamina. Over a period of 10–21 days, hundreds of parasites develop within cysts, which eventually rupture, releasing parasites into the hemocoel where they traffic to the salivary glands. Helminths invade the hemocoel and migrate to the head but not the salivary glands. Rather, worms break out of the proboscis during feeding and infect the vertebrate host from the skin.

PATTERN RECOGNITION RECEPTORS AND PROTEASE CASCADES

PGRPs and GNBPs for Detecting Infection

Peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs) are PRRs that detect Gram-positive (Gram +) and Gram-negative (Gram−) bacteria and fungi, leading to intracellular signal transduction (Figure 2).[18–20] Peptidoglycan (PGN) is an essential component of bacterial cell walls consisting of β-(1–4)-linked *N*-acetylglucosamine and *N*-acetylmuramic acid polymers cross-linked by short peptides. L-Lysine is found at the third position of the peptide chain in most Gram+ bacteria, whereas Gram− bacteria and Bacilli have *meso*-diaminopimelic acid (DAP) at this position. The PGRP PGN binding domain (~165 amino acids) is homologous to bacteriophage T7 lysozyme, a Zn^{2+}-dependent amidase that hydrolyzes the amide bond between *N*-

acetylmuramic acid and L-alanine of PGN, but most PGRPs lack amidase activity because of the absence of a key cysteine for Zn coordination.

Sensing of Gram+ bacteria in the hemolymph is mediated by a complex of PGRP-SA and GNBP1. GNBP1 hydrolyzes Lys-type PG to fragments recognized by PGRP-SA. Fungal pathogens are separately detected by GNBP3. Both PGRP-SA/GNBP1 (Gram+) and GNBP3 (fungi) activate a protease cascade culminating in cleavage of the cysteine knot protein Spätzle (SPZ).[21] Cleaved Späztle binds to the cell surface receptor Toll, inducing receptor dimerization and signaling.

A separate system of PGRPs activates the Imd pathway in response to Gram− bacteria. DAP-type PG and 1.6-anhydro MurNac are recognized by PGRP-LC, a transmembrane receptor. PGRP-LC has three splice isoforms (LCa, LCx, and LCy) that heterodimerize upon binding tracheal cytotoxin (TCT) to induce signaling. The structures of the PG domain of PGRP-LE and PGRP-LCx in complex with TCT reveal that the discrimination between DAP-type and Lys-type PGN is achieved by an arginine residue found in both proteins. PGRP-SD, initially reported to detect certain Gram+ bacteria, also binds DAP-PGN was recently shown to enhance activation of the Imd pathway.[22]

PGRP-LC signaling is enhanced by PGRP-LE. Full-length PGRP-LE is localized cytoplasmically where it senses intracellular pathogens, while a truncated form of PGRP-LC is localized extracellularly where it binds and recruits TCT to PGRP-LC. Other PGRPs inhibit the Imd pathway, such as the transmembrane receptor PGRP-LF that inhibits signaling by interacting with PGRP-LC. Finally, catalytic PGRPs such as PGRP-LB act as scavengers by hydrolyzing PGN to suppress immune responses.

CLIP Proteases in Sensing and Effector Pathways

Proteolytic cascades in vertebrates regulate complement activation and blood clotting. A similar role is played by the arthropod-specific family of CLIP serine proteases (SP).[23,24] CLIPs comprise an N-terminal disulfide-rich “clip” domain (resembling a paper clip),[25,26] a linker of variable length, and a C-terminal chymotrypsin-like protease domain.[27–29] The clip and SP domains may be tightly or weakly associated. Phylogenetic analysis has identified four clades in arthropod CLIPs, CLIPA–D.[30] CLIPAs are serine protease homologues (SPHs) in which the catalytic serine is mutated. CLIPB–D SPs are principally distinguished by homology within the clip domain. CLIPs are secreted as inactive zymogens and activated by proteolysis in a conserved loop near the N-terminus of the SP domain.

A CLIP SP cascade regulating Toll signaling in response to Gram+ bacteria and fungi has been identified in *D. melanogaster*, the mealworm beetle *Tenebrio molitor*, and the tobacco hornworm *Manduca sexta*. The nonclip protease ModSP is activated by PGRP-SA/GNBP1[31] and in turn activates CLIPB SP Grass (*D. melanogaster*),[32] SAE (*Te. molitor*),[33] or HP6 (*M. sexta*).[34] Grass cleaves the CLIPC SP *Spirit*,[35] though *spirit* mutants retain Toll activity (B. Lemaitre, personal communication). The proteolytic cascade terminates with CLIPB SP Spätzle-processing enzyme (SPE) (*Drosophila* and *Te. molitor*)[36,37] or HP8 (*M. sexta*), which cleaves the Toll ligand Spätzle. SPE can be independently activated by the CLIP SP

Persephone in response to fungal infection.[38,39] Persephone is activated by "danger signals" such as proteases secreted by pathogens or indicators of cell stress.[32,40]

Another CLIP cascade regulates the melanization response to infection and injury, culminating in the conversion of PPO to PO. *D. melanogaster* PPO2 is activated by the CLIPB SPs Sp7/MP2 and MP1,[41–43] or a CLIPC SP, Hayan, specifically in response to wounding.[44] Orthologous PPO-activating CLIPB SPs are SPE (*Te. molitor*),[37] PAP1–3 (*M. sexta*),[45,46] CLIPB9 (*A. gambiae*),[47] IMP-1 (*Aedes aegypti*),[48] and PPAF-I (*Holotrichia diomphalia*).[27,28] Some additional upstream CLIPs in the melanization cascade are known. *A. gambiae* CLIPB8 participates in the melanization cascade but cleaves neither CLIPB9 nor PPO directly.[49] *M. sexta* HP6 cleaves PAP1,[34] and HP21 cleaves PAP2 and −3.[50] H. *diomphalia* PPAF-III acts upstream of PPAF-I.[51]

CLIPA SPHs also play key roles in CLIP cascades despite not being active proteases. Cleavage of *H. diomphalia* CLIPA SPH PPAF-II by PPAF-III or *Te. molitor* SPH1 by SAE results in the formation of an oligomeric complex with PPO.[27,37] PPAF-II is cleaved in the penultimate loop of the CLIP domain. Disulfide bonds prevent dissociation of the PPAF-II clip domain,[27,51] unlike the CLIP domain of PPAF-I. In *M. sexta*, SPHs are required for efficient cleavage of PPO by PAP-1.[52] Thus, CLIPA SPHs act as proteolysis-inducible binding partners to regulate CLIP proteolytic cascades.

Anopheles genomes have 53–95 CLIPs[53] that are involved in lysis and melanization of Gram− bacteria, Plasmodium parasites, and entomopathogenic fungi.[54,55] Three CLIPA SPHs–CLIPA2, CLIPA5, and CLIPA7–are inhibitors of melanization, while CLIPB3, CLIPB4, CLIPB8, CLIPB17, and SPH CLIPA8 are required for robust melanization. Nevertheless, the majority of CLIPs, of which a typical insect has dozens, remain uncharacterized.

MOLECULAR EFFECTORS OF IMMUNITY

Insects have several signaling pathways for detecting infection. The successful detection of infection initiates the Toll, Imd, JNK, and JAK/STAT signal transduction cascades. This results in multiple systemic responses to counter infection.

Antimicrobial Peptides (AMPs)

The most well-studied systemic response is secretion of antimicrobial peptides (AMPs). Dozens of AMP genes in four structural classes have been identified in insect genomes targeting Gram+ bacteria, Gram− bacteria, and fungi.[56] While AMP gene families show high rates of gene duplication and deletion, they do not typically exhibit rapid evolution compared to that of other immune genes, suggesting they are subject to balancing selection whereby pathogens adapt to the most common genotypes within a population.[57] AMPs are generally small (<10 kDa), cationic, amphipathic peptides that bind to and disrupt microbial cell membranes. Multiple mechanisms of action have been identified, including pore formation/membrane depolarization,[58] binding lipid II,[59,60] and inhibition or delocalization of membrane proteins.[61]

Insect Thioester-Containing Proteins (iTEPs)

Thioester-containing proteins (TEPs) make up a superfamily of 150–200 kDa secreted proteins, containing an intramolecular β-cysteinyl–γ-glutamyl thioester bond, that function as effector arms of innate immunity (Figure 3).[62] Arthropod TEPs have two well-studied vertebrate counterparts, complement factors and α_2-macroglobulins (A_2Ms).[63,64] Complement factors are deposited on pathogen surfaces where they enhance phagocytosis (opsonization), recruit immune cells (chemotaxis), and direct the lysis of pathogens. A_2Ms are pan-protease suicide inhibitors that sequester proteases, inhibiting their activity toward macromolecular substrates. The thioester mediates covalent attachment of a TEP to its substrate, though in some TEPs the actual thioester motif is mutated. Complement factors and A_2Ms are found in crustaceans and arachnids but not insects, while arthropods have two distinct TEP families: insect TEPs (iTEPs) and macroglobulin/complement-related (MCR).

The structure of iTEPs comprises eight macroglobulin domains (MG1–8), with nested insertions of a β-sheet domain (CUB), and the α-helical thioester domain (TED) between MG7 and MG8. The thioester bond is sequestered from the solvent by a protein–protein interface between TED and MG8. A large conformational change triggered by cleavage in a protease-sensitive region within the MG6 domain exposes the thioester in the direct proximity of a substrate. The best-studied iTEP is TEP1 from the malaria vector *A. gambiae* (TEP1),[65] a structural homologue of complement factor C3.[66] TEP1 opsonizes Gram− bacteria and targets *Plasmodium* parasites for lysis. Distinct TEP1 alleles are found in *A. gambiae* strains that are susceptible (S) or refractory (R) to *Plasmodium* infection.[67,68]

A major difference between complement factors and iTEPs is the anaphylatoxin domain (ANA). ANAs are cytokines that act as molecular wedges in the inactive conformation of the complement, stabilizing the TED–MG8 interface. Activation by a specific protease complex, known as a convertase, releases ANA and induces a massive conformational change that exposes the thioester bond. In place of ANA, TEP1 is regulated by the leucine-rich-repeat (LRR) proteins LRIM1 and APL1C,[69,70] members of the mosquito-specific LRIM family.[71] LRIM1 and APL1C form a heterodimeric complex via C-terminal coiled-coil domains with an interposed helix–loop–helix motif.[72] Cleavage of TEP1 in the protease-sensitive region produces $TEP1_{cut}$, which requires the LRIM1/APL1C heterodimer for stability both *in vitro* and *in vivo*.[73,74]

The $TEP1_{cut}$/LRIM1/APL1C complex is proposed to be recruited to surfaces where $TEP1_{cut}$ is activated and attaches to the substrate, initiating a positive feedback of TEP1 deposition and effector immune responses. The CLIP SPH SPCLIP1 is required for accumulation of TEP1 on microbial surfaces.[75] In contrast, CLIPA2 is recruited to microbial surfaces but inhibits accumulation of additional TEP1.[76] SPCLIP1 and CLIPA2 are proposed to interact with a TEP1 “convertase”, a pivot point between immune recognition and effector responses.

Far less is known about the role of iTEPs in other insects. *Ae. aegypti* TEP2 limits infection with Dengue virus (DENV), an emerging infectious disease of major global significance.[77] *D. melanogaster* contains four iTEPs (*Tep1–4*) with a canonical thioester motif.[78] *Tep1–4* are upregulated in the fat body following immune stimulation. The TEPs appear to be

differentially involved in directed immune responses to a wide variety of pathogens. *Tep2* knockdown (kd) in *Drosophila* S2 cells reduces the phagocytosis of *Escherichia coli*, while *Tep3* kd reduces the phagocytosis of *C. albicans*;[79] *Tep2–Tep4* ko in flies did not result in reduced phagocytosis or survival for a number of pathogens, suggesting iTEP function is dependent on the infection model.[80] For instance, *Tep2* and *Tep4* ko flies show impaired survival relative to that of wild-type flies when challenged with *Porphyromonas gingivalis*,[81] and *Tep3* reduces mortality from infection by the entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora*.[82]

Transglutaminases

Transglutaminases (TGs) catalyze the deamidation and transamidation of glutamine, cross-linking of proteins by formation of ε-(γ-glutamyl)lysine isopeptide bonds, and play vital roles in blood clotting, regulating cellular responses to stress, and formation of the epithelium.[83] Most insects, including *Drosophila* and *Aedes*, have a single TG that promotes hemolymph coagulation upon injury, trapping pathogens entering through the cuticle.[84] TGs are also involved in cuticle morphogenesis; *D. melanogaster* kd has an abnormal morphology and a pupal semilethal phenotype.[85]

D. melanogaster TG kd also leads to a significant reduction in life span in adults reared under normal, but not germ-free, conditions.[86] Interestingly, the latter effect is proposed to result from a lack of immune tolerance to commensal bacteria rather than suppression of immune responses to infection. *Culex* and *Anopheles* share a second TG. The role of the second TG in *Culex* and *Anopheles* is still ambiguous. However, *A. gambiae* TG2 was recently shown to be upregulated by wounding and involved in injury-induced immune responses that are cross-reactive against human malaria,[87] suggesting its role is similar to that of *A. gambiae* TG1.

EPITHELIAL AND CELL-MEDIATED IMMUNE RESPONSES

Reactive Oxygen Species (ROS) Signaling

Pathogenic invasion or injury of barrier epithelia leads to the generation of reactive oxygen species (ROS) by the Nox/Duox family of oxidases. *Drosophila* Duox is responsible for limiting microbial proliferation in the gut,[88] stimulates epithelial renewal,[89] and promotes cuticle formation by catalyzing the cross-linking of tyrosine.[90] Duox expression and activity are “fine-tuned” to induce ROS in the case of infection yet limit oxidative damage under physiological conditions.[91] In the malaria vector *A. gambiae*, Duox and the peroxidase HPX15/IMPer act as negative regulators of the immune response; RNAi kd of Duox and IMPer results in a significant reduction in the extent of *Plasmodium* infection.[92]

Invasion of *Plasmodium* parasites damages epithelial cells, resulting in nitric oxide synthase (NOS) and peroxidase, NO production, and ultimately apoptosis.[93,94] NOS has been proposed to act as a chemical “time bomb” for the host cell that is toxic to the invading parasite,[95] but evidence suggests most parasites are killed in the extracellular space between the epithelium and the basal lamina.[96] However, epithelial nitration modifies *Plasmodium* surface proteins on parasites that are subsequently targeted for lysis by TEP1.[97] RNAi

silencing of the peroxidase HPX2 or NADPH oxidase NOX5 eliminates subsequent lysis of parasites. Furthermore, African *Plasmodium falciparum* parasites evade destruction by expressing a surface protein Pfs47,[98] which acts to suppress JNK signaling that regulates HPX2/NOX5-mediated nitration.[99]

Phagocytosis and Opsonization

ROS signaling arising from epithelial wounding also attracts hemocytes that rapidly commence phagocytosis of invading bacteria.[100] While plasmatocytes or granulocytes are generally phagocytic, a wide range of immune effectors influence phagocytosis.[101,102] Opsonins bind directly to pathogens to enhance phagocytosis; known arthropod opsonins include iTEPs,[103] lectins,[104] fibrinogen-related proteins (FREPs),[105] and the hypervariable IgG domain protein Dscam.[106,107]

Opsonins are detected by cell surface receptors on hemocytes, some of which are orthologs of vertebrate macrophage receptors. The putative receptor for TEP1 is an ortholog of mammalian A_2M receptor LRP1,[103] while another family, scavenger receptor class B, consists of orthologs of mammalian CD36.[108] Arthropod-specific receptors include scavenger receptor class C (Sr-C) and the EGF-repeat receptors Eater and Nimrod.[109,110] Hemocytes may also phagocytose fragments of self-tissues, termed autophagy, stimulated by the distinct EGF-repeat receptor Draper,[111,112] an ortholog of *Caenorhabditis elegans* CED-1 and mammalian MEGF10.

Macroglobulin Complement-Related (MCR)

The arthropod TEP MCR is highly conserved with almost exclusively a single gene and strict 1:1 orthologs throughout Insecta. Compared to other TEPs, MCR has insertions within the N-terminal chain, an LDLa domain in place of the complement ANA, a mutated thioester motif, and a C-terminal transmembrane helix in most known sequences. The closest vertebrate ortholog is CD109, a GPI-linked TEP localized to the surface of thrombocytes, activated T-cells, and endothelial cells that serves as a negative regulator of TGF-β signaling.

D. melanogaster Mcr has an essential role in epithelial development, the proper formation of septate junctions between cells, without which the epithelium is malformed and permeable.[113,114] Mcr is also reported to function in hemocytic immune responses. Mcr is expressed in plasmatocytes. *Mcr* kd in S2 cells led to a defect in the phagocytosis of *C. albicans*,[79] although no phenotype was observed when *dsMcr* flies were challenged *in vivo*.[80] Notably, hemocytes form septate junctions during the process of encapsulation, where layers of cells form an impermeable barrier around a foreign body too large to be phagocytosed.[115] Thus, it seems likely that MCR, like Toll, plays a dual role in both development and immunity.

ANTIVIRAL IMMUNITY

Arboviruses, viruses transmitted to humans via arthropod vectors, are of great historic and topical significance, including yellow fever (YFV), Dengue (DENV), Japanese encephalitis (JEV), Rift Valley fever (RFV), West Nile virus (WNV), O'nyong'nyong (ONNV),

Chikungunya (CHIKV), and most recently Zika (ZIKV). Most arboviruses are (+)ssRNA viruses of the genera *Flavivirus* (YFV, JEV, DENV, WNV, and ZIKV) and *Alphavirus* (ONNV and CHIKV). The dominant arbovirus vectors are mosquitoes of the genus *Aedes* (primarily *Ae. aegypti* and *Aedes albopictus*) and *Culex*, while anopheline mosquitoes are primary vectors only for ONNV. Ticks are vectors for several arboviruses, mainly tick-borne encephalitis (TBE).

The main immune response of insects to arbovirus infection is from small interfering RNA (siRNA).[116] Small viral RNAs are found in infected *Ae. aegypti*,[117] and silencing of siRNA components—*Dcr2*, *Ago2*, *TSN*, and *r2d2*—increases viral titers in *Ae. aegypti* and *A. gambiae*.[118,119] Infecting *Ae. aegypti* with recombinant Sindbis containing DENV RNA protected them from subsequent DENV infection. A second, Dicer-independent, form of RNAi is Piwi-interacting RNA (piRNA), 24–30-nucleotide small RNAs with a bias for 5′U on antisense and 10A on sense strands that restricts transposon activity. The Piwi gene family is greatly expanded in arbovirus vectors *Aedes* and *Culex* compared to *Drosophila*. piRNAs are detected in virus-infected *Ae. aegypti* cell lines and mosquitoes.

The importance of RNAi-mediated immunity is evidenced by the rapid evolution and positive selection of genes in RNAi pathways.[120] Arboviruses infect only a fraction of the population and so are unlikely to be common drivers of selective pressure, but genetic diversity does correlate with lower vector competence in the field.[121] Flaviviruses can suppress RNAi immune responses in the host through transcription of a small structured noncoding RNA, sfRNA.[122] Arboviruses can persist in mosquitoes either as a latent infection during diapause or by transovarial transmission to future generations. Despite lacking reverse transcriptases, it has recently been shown that arboviruses may even persist by integration into host genomes through the action of endogenous reverse transcriptases.[123]

Viral infection also leads to activation of other effector immune responses such as the JAK-STAT pathway,[124] leading to secretion of antiviral effectors such as the interferon-like cytokine Vago,[125] and AMPs.[126] TEPs also play a role in antiviral immunity. *Drosophila* employs transcriptional pausing to regulate a rapid antiviral immune response, including rapid transcription of *Tep2*.[127,128] *Ae. aegypti* TEPs, and specifically *Ae. aegypti* MCR, restrict Dengue infection via an interaction with scavenger receptor class C (SR-C).[77,129]

FERTILITY, LONGEVITY, AND IMMUNOLOGICAL MEMORY

The arthropod immune system changes with age, an important factor given infectious vectors are significantly older than the population average. RNA transcripts upregulated in *Drosophila* at 4 weeks of age versus 1 week of age are enriched with immunity-related genes, including PGRPs, the NF-kB homologue Relish, and effector genes such as AMPs and *Tep4*.[130] Upregulation of *Drosophila* immune genes is observed upon mating,[131,132] yet in general, immunity trades off with reproductive output.[133] An age-related decline in *Anopheles* fecundity is correlated with reactive oxygen species, countered by administration of antioxidants and catalase activity.[134]

This trade-off occurs at the molecular level where proteins play dual roles in development, immunity, and/or fertility. *A. gambiae* TEP1 targets not only pathogens but also damaged spermatogonia in the male.[135] TEP1 alleles associated with higher fertility are associated with increased susceptibility to *Plasmodium*. This suggests that common danger signals arise from damaged spermatogonia and epithelial damage from invasion by malaria parasites and moreover that selective pressure to maximize male fertility might have undesired consequences on the vectoral capacity of females for malaria.

Activation of innate immunity causes long-lasting changes that can protect insects from future infection. This is explained by two related immunological concepts once considered exclusive to adaptive immunity. Immune priming is enhancement of the immune response to infection by prior immune system activation.[136] Immune memory is the ability of the immune system to store or use information on a previously encountered pathogen,[137] implying a degree of specificity in the secondary response.[138,139] Immune priming by bacteria causes hemocyte differentiation in *Anopheles* mosquitoes that suppresses subsequent *Plasmodium* infection,[140] mediated by a soluble factor within the hemolymph identified as a lipoxin/lipocalin complex.[141] Gut microbiota also activate the immune responses of *Aedes* to arboviruses.[142] However, priming comes at a cost for fecundity,[143] again suggesting that selective pressure to reproduce has undesirable consequences for vectoral capacity.

Priming is actually required for immune system development. The tsetse fly *Glossina morsitans* harbors the maternally transferred endosymbiont *Wigglesworthia* and commensal *Sodalis*. Flies raised under aseptic conditions are immunocompromised as evidenced by reduced populations of hemocytes, a lack of ROS/iTEP/AMP induction upon infection, and increased susceptibility to bacteria and trypanosomes.[144,145] *Drosophila* hemocytes are primed by autophagy of cell corpses during larval development, mediated by Ca^{2+}-triggered JNK signaling and the cell death receptor Draper.[146] The obligate endosymbiont *Wolbachia* is common in numerous insects, and the engineered life-shortening strain wMelPop primes *Ae. aegypti* for refractoriness to Dengue, Chikungunya, and Zika virus, *Plasmodium gallinaceum*, and filarial nematodes.[147–149] Natural and deliberate *Wolbachia* infection stimulates immune gene expression in anopheline mosquitoes and reduces vectoral capacity for *Plasmodium*.[150–152]

Priming does not fully explain the role of *Wolbachia* infection, however, because in its natural host *Drosophila* wMelPop does not lead to Toll or Imd activation, yet infection still induces an antiviral response.[153–155] Indeed, the presence of *Wolbachia* blocks viral genome replication without evidence of a transcriptional response in the canonical siRNA pathway.[156] Other potential mechanisms for the immune enhancement of *Wolbachia* are resource competition, cytoplasmic remodeling, and other intrinsic factors.

CONCLUSION

Several strategies for translating the expanding knowledge of arthropod innate immune responses to reduce the rate of transmission of human pathogens are being explored. One approach is the use of entomopathogens for biological control. *Bacillus thuringiensis*

israelensis (Bti) is a broad-spectrum and cost-effective mosquito larvicide.[157,158] Fungal biopesticides are also being investigated for control of mosquitoes, triatomes, and ticks.[159–162] A second effort is focused on developing transgenic mosquitoes refractory to malaria transmission by the expression of anti-*Plasmodium* factors, blocking invasion of the midgut of salivary gland epithelia, or manipulation of the mosquito immune system.[163] Given the known trade-off among immunity, longevity, and fertility, the spread of a transgene through wild populations is a significant hurdle; this can be overcome with gene drive mechanisms.[164]

Paratransgenesis, the modification of symbiotic organisms to deliver anti-pathogen effector molecules, combines the strategies described above using viral, bacterial, and fungal pathogens.[165–167] *Wolbachia* in particular, besides its immune effect, contains an intrinsic genetic drive mechanism, and life-shortening strains of *Wolbachia* reduce population life span, effectively preventing transmission by eliminating the older, infectious members of the population. This approach has proven effective in multiple field trials with negligible risk.[168,169]

Nevertheless, there are numerous questions outstanding. Many humoral immune factors—PGRPs, GNBPs, CLIPs, etc.—remain uncharacterized. Given the many species-specific expansions in these gene sets, it will be a significant task to determine functional redundancy and crosstalk in extracellular sensing and effector pathways. Similarly, the function of iTEPs is largely undescribed outside *A. gambiae*; the precise function of LRIMs is not known, nor have their orthologs (if any) outside mosquito genomes been identified. The dual roles of iTEPs and MCR in development and immunity remain to be elucidated. While biopesticides, transgenesis, and paratransgenesis are promising strategies for translating knowledge from lab to field, in the future, advances may result in the development of small molecules (e.g., lipoxin derivatives) that stimulate arthropod immune systems to block disease transmission. If these strategies can be deployed as part of an integrated disease eradication program, we may look forward to the day when bug bites are just a nuisance, not a deadly threat.

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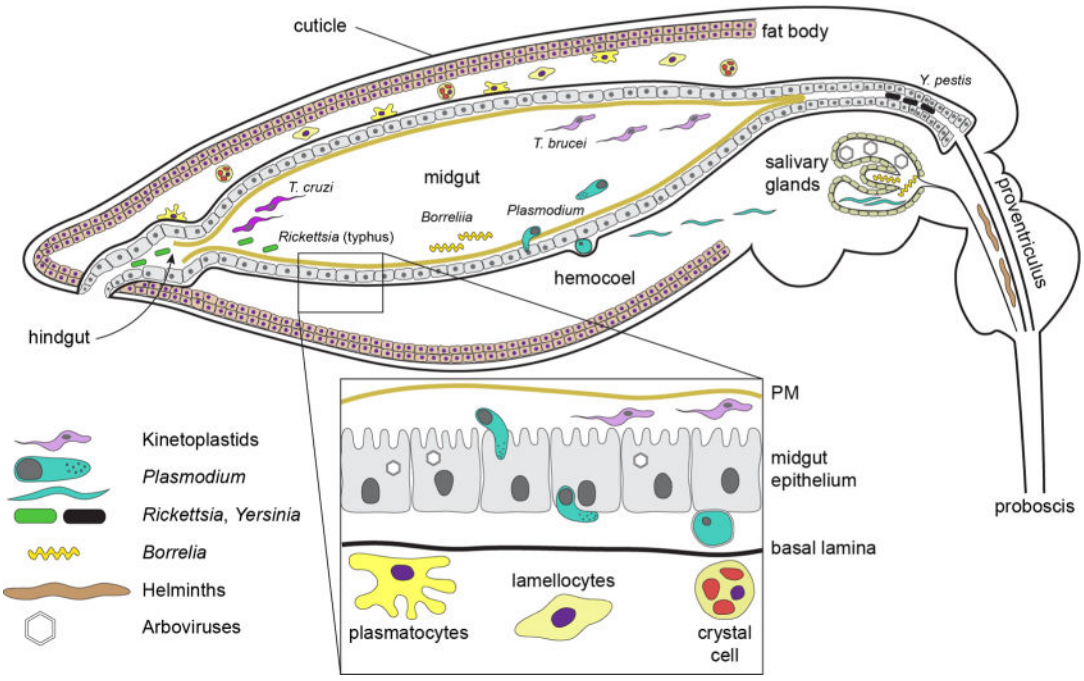

Figure 1.

Physiology of the insect immune system and infection by different disease vectors. Kinetoplastids and some bacteria live in the gut lumen; *T. brucei* and *Y. pestis* infect a new host by regurgitation, while *T. cruzi* and typhus are excreted onto the new host's skin. Arboviruses infect cells of the gut epithelium and progress to the esophagus and eventually the salivary glands. *Plasmodium* parasites traverse the epithelium and form oocysts, from which they emerge and invade the salivary glands from the hemocoel. *Borrelia* spirochetes also invade the salivary glands. Helminths invade the insect head parts, exit the cuticle, and infect new hosts by the exterior of the proboscis.

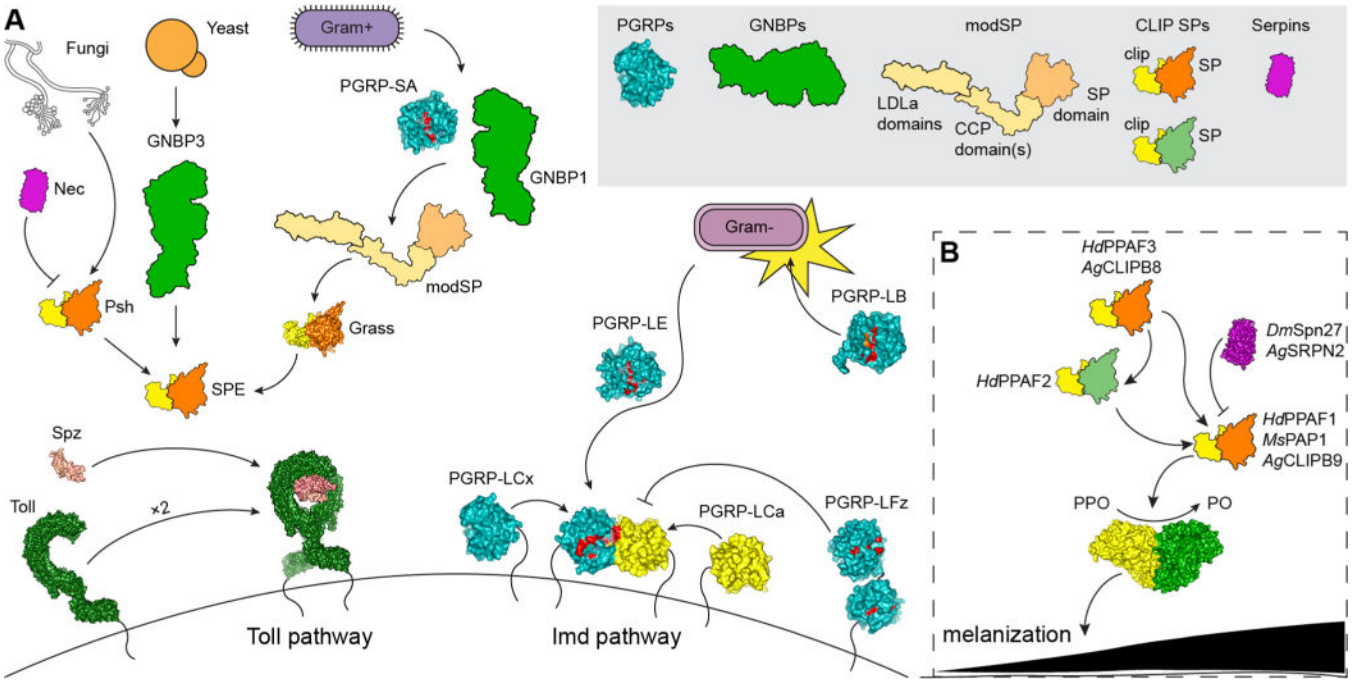

Figure 2.

Pattern recognition receptors and protease cascades. Proteins of known structure are shown in surface representation and unknown structures in silhouette. (A) Peptidoglycan (Lys-PGN) from Gram+ bacteria is detected by PGRP-SA/GNBP, stimulating a CLIP protease cascade terminating with Spätzle-processing enzyme (SPE). Yeast and fungi independently activate SPE via GNBP3 and Persephone (Psh), respectively. SPE cleaves the cysteine knot protein Spätzle, which dimerizes and binds the LRR receptor Toll. Peptidoglycan (DAP-PGN) from Gram− bacteria is detected by the membrane receptors PGRP-LC. The PGRP-LCa/LCx heterodimer detects the PGN fragment TCT; PGRP-LCx homodimers detect polymeric DAP-PGRN. PGRP-LE binds TCT, enhancing signaling, while PGRP-LFz inhibits PGRP-LC dimerization. (B) The final elements of the protease cascade terminating in CLIPB-dependent activation of PPO are conserved in flies, mosquitoes, beetles, and moths. Numerous CLIPAs, CLIPBs, and serpins regulate melanization cascades but are less conserved among insects.

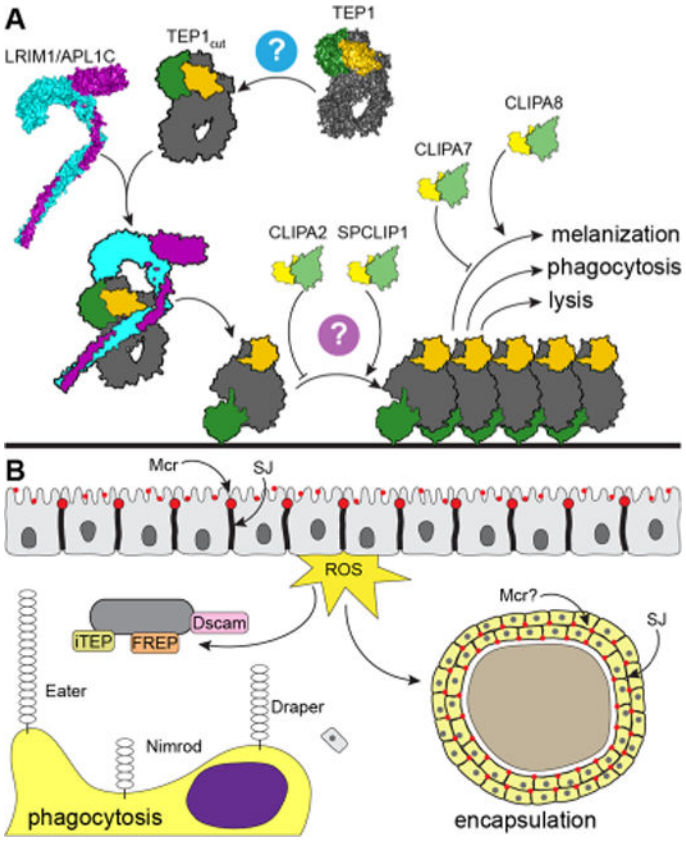

Figure 3.

Thioester-containing proteins and epithelial/cellular immunity. (A) *A. gambiae* TEP1 is cleaved to $TEP1_{cut}$ in the hemolymph and stabilized by a heterodimeric complex of proteins LRIM1 and APL1C. Activation of $TEP1_{cut}$ results in deposition on a pathogen surface. The CLIPA SPHs CLIPA2 and SPCLIP1 act to inhibit and promote, respectively, further deposition of TEP1 via a putative convertase. CLIPA7 and CLIPA8 inhibit and promote, respectively, melanization downstream of TEP1 deposition. Unknown proteases are indicated by a question mark. (B) Epithelial injury results in ROS signaling that promotes local activation of iTEP immune responses and chemotaxis of hemocytes. Pathogens are opsonized by iTEPs, FREPs, and Dscam, recognized by EGF receptors Eater, Nimrod, and Draper. Foreign particles that too large to engulf are encapsulated by hemocytes. MCR is essential for the formation of septate junctions (SJs) between epithelial cells, is reported to effect *in vitro* phagocytosis of *Candida albicans*, and is anticipated to be involved in SJ formation during encapsulation.

Table 1

Common Vector-Borne Diseases and Their Vectors

pathogen	vector	genus or species
flaviruses		
yellow fever, Japanese encephalitis, West Nile virus Dengue, Zika	mosquitoes	*Aedes*, *Culex*
O'nyong'nyong, Chikungunya		*Anopheles*, *Aedes*
bacteria		
Yersinia pestis (plague)	fleas	*Xenopsylla*
Rickettsia prowazekii (typhus)	lice	*Pediculus humanus*
Rickettsia (e.g., Rocky Mountain spotted fever), *Borrelia* (Lyme disease)	ticks	*Dermacentor*, *Ixodes*
protozoa		
Plasmodium (malaria)	mosquitoes	*Anopheles*
Trypanosoma		
Trypanosoma cruzi (Chagas disease)	kissing bugs	*Rhodnius*, *Triatoma*
Trypanosoma brucei (sleeping sickness)	tsetse flies	*Glossina morsitans*
Leishmania (leishmaniasis)	S flies	*Phlebotomus*, *Lutzomyia*
filarial worms		
Onchocerca (river blindness)	black flies	*Simulium*
Loa loa	deer flies	*Chrysops*
Wucheria bancrofti, *Brugia malayi*	mosquitoes	*Culex*, *Anopheles*, *Aedes*, *Mansonia*