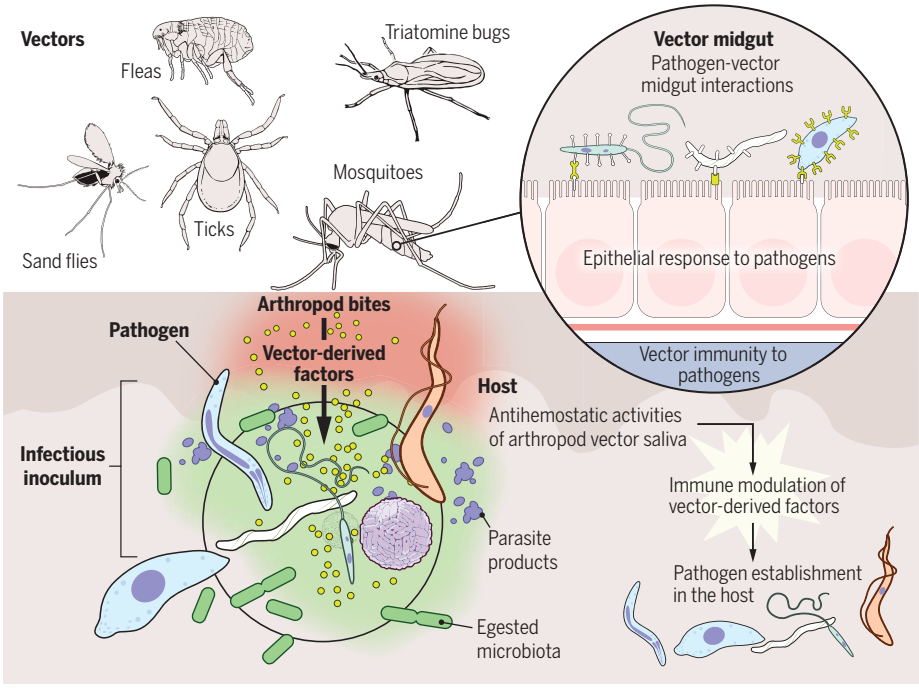
REVIEW SUMMARY

HUMAN DISEASE

Understanding pathogen survival and transmission by arthropod vectors to prevent human disease

Carolina Barillas-Mury*†, José M. C. Ribeiro*†, Jesus G. Valenzuela*†

BACKGROUND: Many endemic human and animal diseases caused by viruses, bacteria, protozoa, or nematodes are transmitted by blood-feeding insects or ticks. Because pathogens are ingested with the blood meal, their interactions with the vector's gut microbiome, midgut secretions, and epithelial cells are key determinants of disease transmission. Most pathogens must infect the vector and multiply to be transmitted, and this amplification greatly increases their chances of infecting a new vertebrate host. Accordingly, how pathogens survive or invade the midgut, and their interactions with the invertebrate immune system, are areas of great interest in vector biology. Blood-feeding arthropods secrete saliva while probing for blood, and saliva from many different vectors has been shown to have antihemostatic, anti-inflammatory, and immunomodulatory activities that allow successful blood feeding. Other vector-derived factors, including exosomes, egested microbiota, and pathogen-derived molecules, are injected at the bite site where pathogens are delivered and modulate the local environment and the innate immune responses of the host skin, promoting pathogen establishment and disease progression. Thus, functional studies of vector saliva and other vector-derived factors, their roles in pathogen establishment and transmission, the potential use of specific protein targets to prevent vector-borne diseases, and the use of vector salivary proteins as biomarkers of exposure to vector bites are also of great importance.



Interaction of pathogens with arthropod vector molecules promotes pathogen survival and transmission. Vector-borne pathogens interact with the gut microbiota and with secreted molecules and midgut receptors necessary for survival or for cell invasion. This allows them to circumvent the vector immune system to avoid elimination, multiply, and be transmitted. Pathogens also interact with vector-secreted molecules that are coinjected into the skin of the vertebrate host during disease transmission by the bite of an infected vector. These vector-derived factors—which include salivary proteins, parasite exosomes, parasite molecules, and egested microbiota—modify the hemostatic and innate immune response of the host skin, resulting in enhanced pathogen transmission.

ADVANCES: Over the past 20 years, the study of vector biology has been revolutionized by many new molecular, cell biology, and genomic tools. For example, the advent of genomics and third-generation sequencing has allowed for high-density de novo transcriptome assembly and single-cell RNA transcriptome analysis, even without a reference genome. Perturbations in vector gene expression in response to infection with specific pathogens can now be studied at a genome-wide level. The expression of recombinant proteins derived from genomics or transcriptomic studies has enabled structure-function studies, the discovery of salivary biomarkers to measure vector exposure in humans, and the generation of specific antibodies for subcellular localization of pathogen receptors or of vector-derived proteins that interact with specific pathogens. Transient gene silencing has revealed the participation of specific genes or signaling pathways in both vector physiology and in vector immunity against pathogens. There have also been great advances in the genetic manipulation of insects with the CRISPR-Cas9 system and the development of gene-driven strategies, such as CRISPR-Cas9–directed homologous repair. The symbiotic proteobacteria *Wolbachia* has been introduced into natural populations of *Aedes aegypti* mosquitoes to reduce viral replication and transmission of dengue, Zika, and Chikungunya with promising results. Such approaches may hold the long-awaited promise of permanently modifying natural vector populations to render them unable to transmit disease.

OUTLOOK: In this Review, we highlight recent progress in our understanding of the basic molecular and cell biology of pathogen interactions with vectors and their microbiomes, as well as the interactions between vector-derived factors and pathogens with their vertebrate hosts during the establishment of infection after a vector bite. We discuss how these advances in the biology of disease transmission have also translated into next-generation strategies to control natural vector populations and to modify them to disrupt their capacity to transmit disease. Moreover, we review the development of salivary biomarkers of vector exposure as a tool for evaluating both epidemiological studies and vector control strategies by measuring vector biting activity in humans. ■

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Many endemic poverty-associated diseases, such as malaria and leishmaniasis, are transmitted by arthropod vectors. Pathogens must interact with specific molecules in the vector gut, the microbiota, and the vector immune system to survive and be transmitted. The vertebrate host, in turn, is infected when the pathogen and vector-derived factors, such as salivary proteins, are delivered into the skin by a vector bite. Here, we review recent progress in our understanding of the biology of pathogen transmission from the human to the vector and back, from the vector to the host. We also highlight recent advances in the biology of vector-borne disease transmission, which have translated into additional strategies to prevent human disease by either reducing vector populations or by disrupting their ability to transmit pathogens.

Blood-feeding insects are vectors of some of the most devastating diseases in low-income areas, such as malaria, Zika, dengue, and leishmaniasis. Vector-pathogen interactions, particularly those mediated by the midgut, the gut microbiota, and the vector immune system, are intense areas of study because they are major determinants of disease transmission by limiting pathogen survival. Molecular interactions between vector-derived factors—such as salivary proteins, exosomes, and egested microbiota—and the mammalian host modulate local skin immunity and are critical for pathogen establishment. Thus, it is very important to understand how saliva and other vectors or pathogen-derived factors affect disease transmission and to understand their potential as vaccine targets to prevent diseases or as biomarkers of vector exposure.

Interaction of pathogens with vector midgut proteins

Ingested pathogens need to survive the harsh environment of the midgut lumen, where blood digestion takes place. The blood meal is surrounded by a chitinous peritrophic matrix (PM), which prevents direct contact between the gut microbiome and epithelial cells (Fig. 1). In *Anopheles gambiae* mosquitoes, the permeability of the ectoperitrophic space (between the PM and the midgut epithelium) is tightly modulated by secretion of an immune-modulatory peroxidase (IMPer) (*1*). This enzyme cross-links proteins in the ectoperitrophic space, allowing commensal bacteria to proliferate in the gut lumen without eliciting epithelial immunity. This also allows early stages of *Plasmodium* to develop within the gut without detection. In tsetse flies, *Trypanosoma brucei* takes advantage of the immature and fluid PM to move into the ectoperitrophic space. There, it colonizes the proventriculus to later establish midgut infection (*2*).

Some pathogens must bind to specific midgut receptors to survive. *Leishmania major* parasites, for example, are covered with lipophosphoglycans that specifically bind to PpGal, a sand fly midgut galectin receptor required for parasite survival and development (Fig. 1) (*3*). The Borrelia surface protein OSP A binds to TROSPA, a receptor in the gut of the *Ixodes scapularis* tick (Fig. 1), an interaction required for tick colonization by spirochetes (*4*). Thus, arthropod midgut receptors are attractive potential vaccine targets to block disease transmission.

Effect of gut microbiota and multiple blood meals in vectorial capacity

Commensal gut bacteria proliferate extensively as the blood meal undergoes digestion and affect pathogen establishment. In *A. gambiae*, the mosquito gut microbiota limits *Plasmodium* infection, and oral antibiotics increase *Plasmodium* survival (*5*). By contrast, the gut microbiota of sand flies is essential for *Leishmania*, and antibiotics inhibit their development into infective metacyclics (*6*). The close interaction of pathogens with the insect gut microbiota has been exploited by using an enterobacterium isolated from wild-caught *Anopheles* to limit *Plasmodium* development in mosquitoes (*7*) and *Chromobacterium* sp. from field-caught *Aedes* to impair *Plasmodium* and dengue virus infection (*8*).

Recent findings show that ingestion of multiple blood meals can enhance pathogen transmission by infected vectors (Fig. 1). When *Leishmania*-infected sand flies take a second blood meal, parasites dedifferentiate into a multiplicative stage called retroleptomonad, which expands the population of infective metacyclics in the vector gut (*9*). Similarly, dengue virus–infected mosquitoes fed a second uninfected blood meal exhibit higher systemic virus dissemination that enhances viral transmission (*10*). This increase in vectorial capacity after the ingestion of blood from a healthy host may explain why disease transmission is so effective in the field.

Insect innate immunity and vector competence

Antiplasmodial responses of anopheline mosquitoes can limit malaria transmission. In *A. gambiae*, these responses involve the sequential and coordinated activation of epithelial immunity by invaded midgut cells (*11*–*15*), microvesicle release by circulating hemocytes (*16*, *17*), and activation of the mosquito complement system (*15*, *18*). Genetic studies have revealed that Pfs47, a protein on the surface of the parasite *Plasmodium falciparum*, mediates immune evasion (*19*, *20*) by interacting with a receptor in the mosquito midgut (*21*). The receptors have diverged in evolutionarily distant mosquitoes (*21*, *22*) and constantly select for parasites with a *Pfs47* haplotype compatible with their receptor (*22*).

Antiviral immunity in *Drosophila* and mosquitoes is mediated by the RNA interference (RNAi) pathway, whereas the Piwi-interacting RNA (piRNA) pathway appears to be active only in mosquitoes (*23*–*25*). Silencing key components of the RNAi pathway in *Aedes aegypti* mosquitoes increases *Dengue* (*26*) and *Sindbis* virus replication (*27*) and, in *A. gambiae*, enhances the infection and dissemination of *O'nyong-nyong* virus (*28*).

piRNAs range in size between 26 and 31 base pairs and have been mostly linked to the silencing of retrotransposons (*29*) and other genetic elements in the germ line (*30*). However, an antiviral role in mosquito cell lines has been proposed, where viral DNAs (vDNAs) are integrated into the *A. aegypti* genome as endogenous viral elements (EVEs) and serve as templates to produce piRNAs (*31*–*33*). EVEs may therefore represent a type of immune memory by providing a record of past infections (*32*). EVE-derived piRNAs are specifically loaded onto Piwi4 and inhibit virus replication (*33*). Inhibition of vDNA in *Aedes albopictus* and *A. aegypti* mosquitoes infected with two arboviruses (*Dengue* or *Chikungunya*) reduces viral small RNAs and results in high susceptibility to viral infection and loss of tolerance. Thus, vDNA is an important mechanism for persistent viral infections in mosquitoes, which makes these insects highly efficient disease vectors (*34*).

Furthermore, overactivation of the JAK–STAT pathway decreases *A. aegypti* susceptibility to dengue virus infection, whereas silencing key

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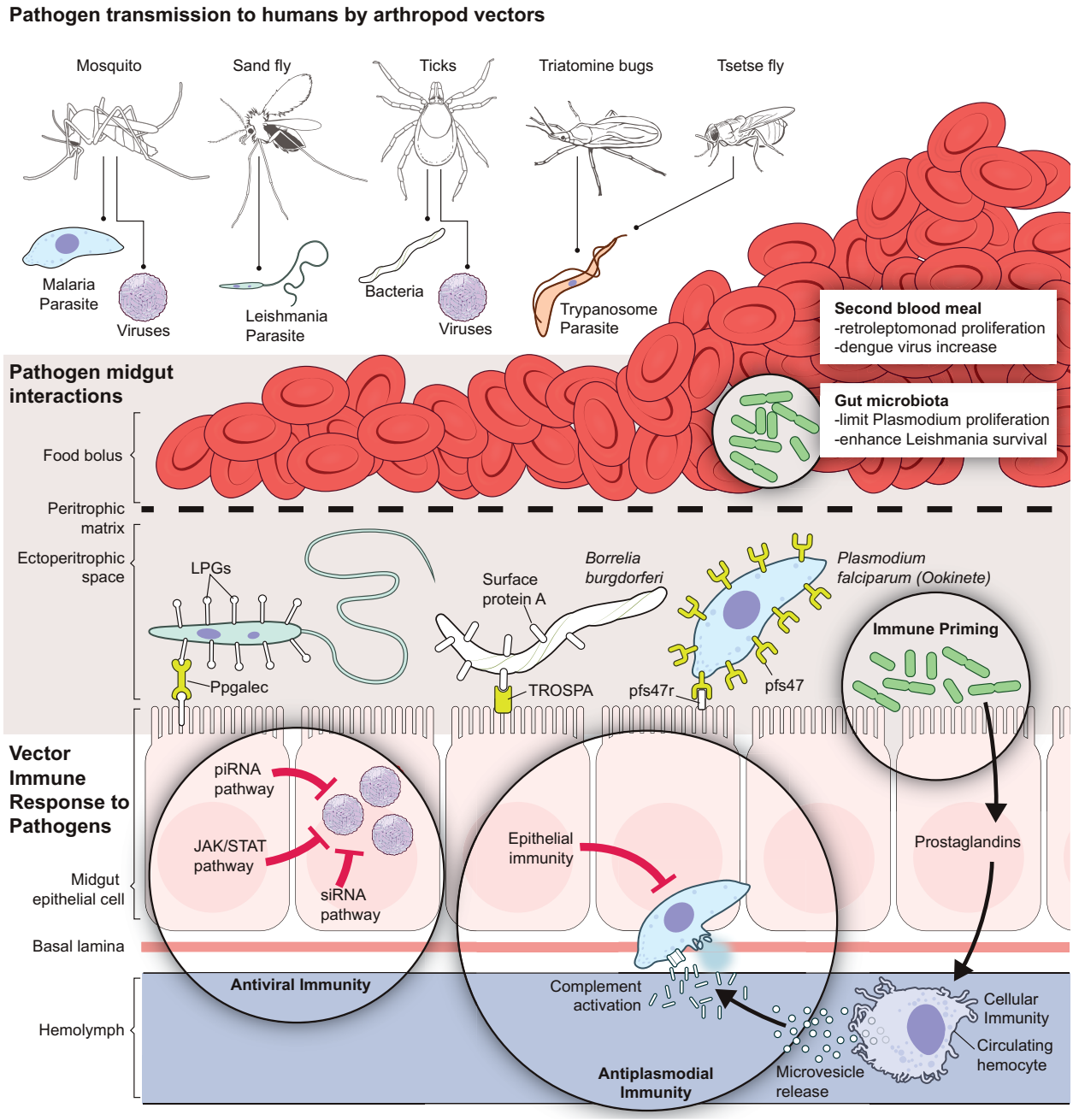

Fig. 1. Pathogens ingested by arthropod vectors interact closely with the gut microbiota and with midgut epithelial cells. They traverse the PM, and some interact with specific receptors, such as *P. papatasi* galectin (PpGal), *I. scapularis* TROSPA, and the *P. falciparum* Pfs47 receptor. Epithelial cells activate antiviral responses mediated by the piRNA, small interfering RNA (siRNA), and JAK–STAT pathways. At the same time, caspase-mediated nitration responses of ookinete-invaded midgut cells activate hemocyte microvesicle release, which promotes the activation of complement-mediated *Plasmodium* elimination. Ingestion of a second blood meal can also provide nutritional and/or differentiation signals that enhance pathogen transmission. LPGs, lipophosphoglycans.

components for activation of the pathway enhances susceptibility to infection (*35*). Activation of the Toll pathway and the gut microbiota reduces *A. aegypti* susceptibility to dengue infection (*36*), whereas constitutive activation of the immunodeficiency (IMD) pathway decreases microbiota bacterial loads and increases Sindbis virus infection (*37*). By contrast, elimination of the gut microbiota in *A. gambiae* decreases infectivity with O'nyong-nyong virus (*38*). The *A. gambiae* midgut activates local antiviral responses to O'nyong-nyong virus infection, which involve the IMD and JAK–STAT signaling pathways, whereas the RNAi pathway mediates systemic responses (*38*). Loquacious 2 (Loqs2) is required to control systemic replication of dengue virus and Zika virus in *A. aegypti*. Loqs2 is not expressed in the midgut, but ectopic midgut expression in transgenic mosquitoes limits dengue virus replication and dissemination (*39*).

Innate immune priming

Insect immunity relies on innate immune responses that were thought to be hardwired. However, studies in *A. gambiae* have revealed that mosquitoes previously exposed to *Plasmodium* infection mount a more effective response to subsequent infections (*1*). The priming response is established when ookinete invasion allows direct contact of the gut microbiota with midgut cells, activating prostaglandin

synthesis mediated by two heme peroxidases (HPx7 and HPx8) (*16*). This triggers the systemic release of a hemocyte differentiation factor (HDF) that increases the proportion of circulating granulocytes (*1*). HDF is a complex of lipoxin A_4 bound to Evokin, a carrier from the lipocalin family. Immune priming involves increased ability of mosquitoes to synthesize lipoxins, predominantly lipoxin A_4 (*40*). Primed hemocytes then release more microvesicles in response to ookinete invasion, which enhances the activation of the mosquito complement system (*17*).

Vector saliva

The saliva of blood-feeding arthropods has antiplatelet, anticlotting, and vasodilatory properties and contains modulators of inflammation and immunity (*41*, *42*) (Fig. 2). Despite similar functions, these proteins are highly divergent among insect species because blood feeding evolved independently, and these genes evolve rapidly. For example, most vectors that feed on mammalian blood have high levels of adenosine 5′-diphosphate (ADP)–hydrolyzing enzymes to inhibit platelet aggregation, but these levels are achieved using different strategies. A 5′-nucleotidase family exists in ticks (*43*), triatomine bugs (*44*), and mosquitoes (*45*); a CD39 family is present in fleas (*46*); and a Cimex-type apyrase is found in sand flies (*47*) and bed bugs (*48*). The most potent vasodilator known, the peptide maxadilan, was discovered in the sand fly *Lutzomyia longipalpis* (*49*). Several "kratagonists" (from the Greek "kratos" meaning "to seize") in vector saliva, such as lipocalins and odorant-binding proteins, act as scavengers of histamine, serotonin, leukotrienes, thromboxane A_2, and inflammatory cytokines (*50*), whereas other protein families inhibit the activation of the complement system (*51*–*54*).

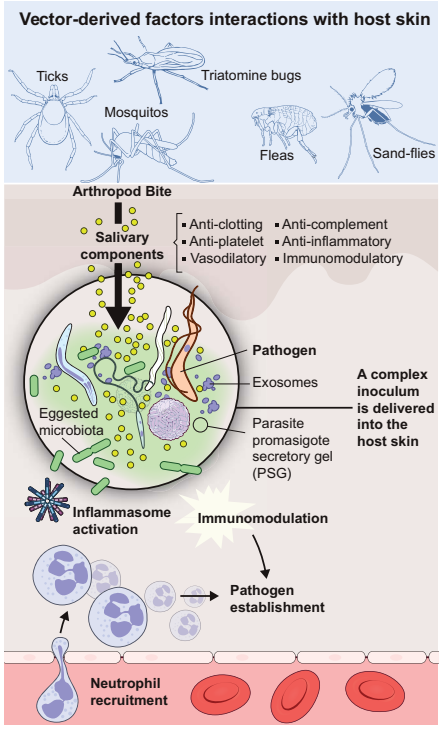

Fig. 2. In addition to pathogens, blood-feeding arthropods deliver a series of vector-derived factors, such as salivary components and gut microbiota, and pathogen components, such as exosomes and proteophosphoglycans, into the host skin. Vector-derived factors counteract the hemostatic system and modulate host skin immunity, including activation of the inflammasome, which favors the establishment of some parasitic and viral infections.

The advent of third-generation sequencing has revealed the existence of many previously unknown salivary proteins—~50 different salivary polypeptides in sand flies, >100 in mosquitoes and triatomine bugs, and thousands in hard ticks (*55*, *56*). The function of most of these proteins remains unknown. The increased number of salivary transcripts in ticks is the result of a large expansion of several protein families. However, only a few members of each family are expressed at a given time, and the composition of saliva changes over time (*57*). This phenomenon, which we refer to as sialome switching, may be an immune evasion strategy, but how it is regulated remains a mystery.

The effect of vector saliva and vector-derived factors on pathogen establishment

Needle inoculation of pathogens does not mimic their natural delivery by an insect bite. For example, West Nile virus delivered to mice by mosquito *Culex tarsalis* bites results in higher viremia and neuroinvasion compared with needle inoculation (*58*). Likewise, injection of Semliki Forest virus in a skin site previously bitten by *Aedes* mosquitoes results in higher viremia and increased mortality in rodents (*59*), mediated by interleukin-1β (IL-1β) activation and recruitment of neutrophils to the dermis (*59*). Similarly, *Leishmania donovani* transmission by sand fly bites induces the activation of the inflammasome, production of IL-1β, recruitment of neutrophils, and parasite visceralization (Fig. 2). By contrast, no parasite visceralization occurs with needle-injected parasites (*60*). Activation of IL-1β and neutrophil recruitment was triggered by the gut microbiota egested during the sand fly bite (*60*). *Plasmodium* sporozoites injected at the bite site by *Anopheles stephensi* result in higher parasitemias, enhanced progression to cerebral malaria, and death (*61*). SALP15, a tick salivary protein, is essential for establishment and dissemination of infection by inhibiting T cell activation and protecting *Borrelia* from the host complement (*62*). Thus, vector-derived factors from different disease vectors appear to activate innate immune responses and to recruit cells, such as neutrophils, that facilitate pathogen establishment (Fig. 2).

There are other mechanisms by which vector saliva facilitates pathogen transmission. For example, the sand fly salivary endonuclease LunDep destroys neutrophil extracellular traps (NETs), increasing survival of *L. major* parasites (*63*). The sand fly salivary hyaluronidase enhances *Leishmania* infection in mice by working as a so-called spreading factor and by increasing neutrophil recruitment to the bite site (*64*, *65*). The *A. aegypti* salivary protein NeSt1 increases *Zika* virus pathogenesis by activating neutrophils (*66*), whereas the salivary protein AaVA-1 enhances *Zika* and *Dengue* transmission in rodents by activating autophagy in immune cells (*67*). In addition to salivary proteins, *Leishmania* proteophosphoglycans and exosomes as well as egested microbiota are part of the infectious inoculum of sand flies (*60*, *68*, *69*).

Arthropod salivary proteins as vaccine targets to prevent disease transmission

Arthropod salivary proteins are promising vaccine candidates to disrupt transmission of vector-borne diseases (*70*, *71*). Vaccination of rhesus macaques with the sand fly salivary protein PdSP15 confers protection from *L. major* transmitted by sand fly bites, which correlates with the development of a PdSP15-specific type 1 T helper (T_H1) cell immunity and anti-*Leishmania* immunity (*72*). T_H1 immunity to the salivary protein coinjected at the bite site is thought to protect from disease by altering the immune response to parasite antigens (*72*). Recently, immunization of guinea pigs with an mRNA vaccine encoding selected tick salivary proteins induced a skin response that prevented *Borrelia* transmission by ticks in vaccinated animals (*73*). In mosquitoes, vaccination of mice with *Anopheles* salivary protein AgTRIO protects animals from *Plasmodium* infection (*74*), whereas immunization with the *A. aegypti* salivary protein AgBR1 protects mice from Zika (*75*) and West Nile virus infections (*76*). Antibodies to AgBR1 prevent AgBR1-induced neutrophil activation and block saliva-mediated enhancement of virus establishment (*75*). Recently, a mosquito salivary peptide–based vaccine was shown to be safe and to produce specific immunity in a phase 1 clinical trial in humans (*77*).

Arthropod salivary proteins as biomarkers of vector exposure

Humans mount antibody responses to certain salivary proteins that can be used as biomarkers of exposure to vector bites (*78*). Several recombinant vector salivary proteins and synthetic peptides have been developed as biomarkers, including the gSG6-P1 salivary peptide from *A. gambiae* (*79*), Nterm-34kDa salivary peptide from *A. aegypti* (*80*), rLJM17 and rLJM11 from *L. longipalpis* (*81*), rSP03B from *Phlebotomus perniciosus* (*82*), PpSP32 from *Phlebotomus*

papatasi (*83*), Tsgf118-43 from tsetse flies (*84*), rTisP14.6 from *Triatoma infestans* (*85*), and Calreticulin from *I. scapularis* (*86*). These are useful tools to assess risk of disease transmission and the effectiveness of vector control strategies (*87*) in ongoing disease elimination campaigns.

Transgenesis, paratransgenesis, and other vector control strategies

Genetic modifications can be used to reduce natural vector populations or to replace them with transgenic vectors that no longer transmit disease (Fig. 3). Two major obstacles to this approach have been overcome: first, the identification of gene targets to be disrupted or previously undiscovered genes that would interrupt pathogen development, and second, strategies to introduce a new gene or to modify a vector gene. However, once released, transgenic insects will mate with wild-type ones, and the genetic modifications of interest will dilute in every generation (*88*) unless they can spread more effectively through non-Mendelian inheritance that can drive the gene through the population.

Several gene drive mechanisms have been proposed, such as transposable elements (*88*) and other gene insertion methods (*89*), including arboviruses and the homing endonuclease system, with various degrees of success. More recently, the CRISPR-Cas9 system has emerged as a very promising approach. CRISPR-Cas9–directed homologous repair of the germ line cuts the gene in the wild-type chromosome of heterozygous mosquitoes and repairs it by homologous repair with the chromosome containing the effector gene of interest (*90*). A great variety of genes have been proposed as insertion targets, including expression of recombinant antibodies that target the pathogen (*91*) or that affect mosquito reproduction (*92*–*94*). However, a word of caution for constructs affecting fertility is needed. A partial decrease of fertility may ultimately lead to more rather than fewer mosquitoes because it will reduce competition for resources during larval stages. Emerging adults may also be better nourished and live longer, resulting in adults with increased vectorial capacity (*95*). This problem can be circumvented by strategies that are deleterious to adult stages but allow normal larval development (*96*).

An alternative strategy to modify vectors is to introduce commensal microorganisms that reduce vectorial capacity (Fig. 3) (*97*). A *Serratia* strain that quickly colonizes the mosquito gut when fed in a sugar solution was recently identified that, when engineered to secrete anti-*Plasmodium* effector proteins, greatly reduces the ability of mosquitoes to transmit malaria (*97*). *Rhodococcus rhodnii*, a triatomine endosymbiont, has been modified to express an anti-*Trypanosoma* peptide. This microbe rendered *Rhodnius prolixus* resistant to *Trypanosoma cruzi*, the parasite that causes Chagas disease (*98*). In this system, symbionts are transmitted from adults to nymphs by coprophagy. Genetically modified *R. rhodnii* has been used to prepare a mixture that is actively ingested by triatomine nymphs (*99*). Another successful development has been the exploitation of *Wolbachia* that naturally infect insects (*100*). This is an intracellular bacterium that is transmitted vertically and spreads through natural insect populations by inducing cytoplasmic incompatibility (CI). Infected females have viable offspring when they mate with either noninfected or infected males, whereas *Wolbachia*-free females are only fertile if they mate with uninfected males. When *A. aegypti* mosquitoes—the vectors of dengue, Zika, and Chikungunya—are infected with some strains of *Wolbachia*, this greatly reduces viral replication and transmission (*101*). This strategy is already undergoing field trials in several countries to disrupt transmission of arboviruses (*102*, *103*). A *Metarhizium* fungus genetically modified to express an insect toxin was very effective in suppressing natural populations of mosquitoes highly resistant to insecticides (*104*). A different strategy, where the antimalarial atovaquone was applied to surfaces with which mosquitoes came into contact, disrupted *Plasmodium* development, providing an additional tool to prevent malaria transmission (*105*).

Future directions

Most blood-feeding arthropods eliminate the pathogens they ingest and do not transmit disease. However, there are extremely rare, fortuitous encounters in which a pathogen interacts with vector factors that allow it to survive and/or evade elimination by the vector immune system and infect a new host when the vector takes the next blood meal. This ability to jump or fly from one host to another gives these pathogens an enormous advantage, with devastating

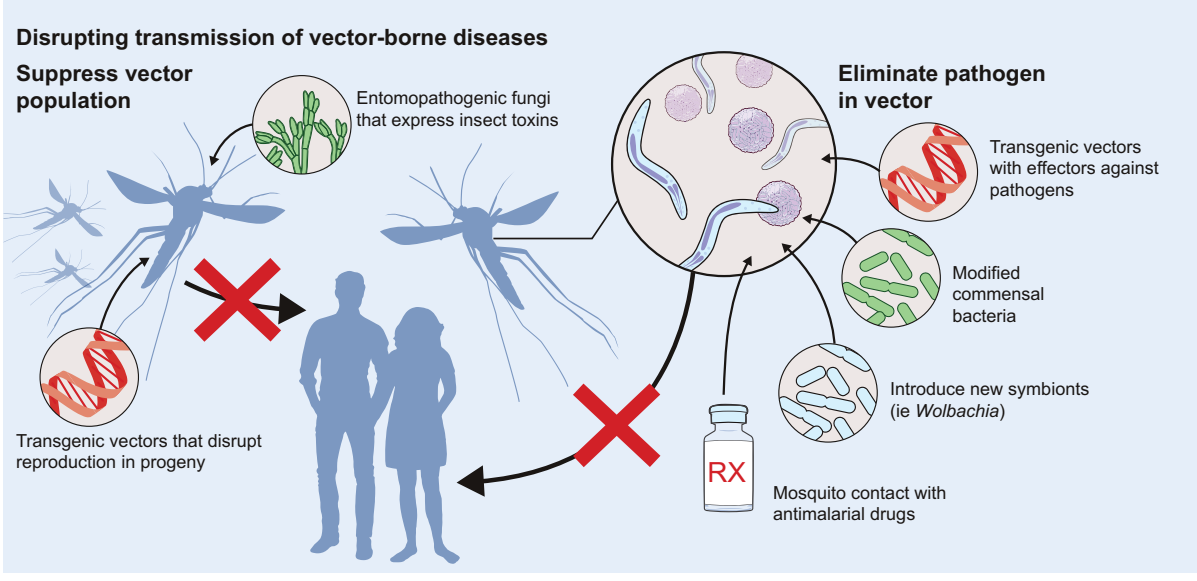

Fig. 3. Next-generation strategies to disrupt pathogen transmission by arthropod vectors include suppression of vector populations or elimination of the pathogens in the vector. Effective entomopathogenic fungi expressing insect toxins or genetic modifications deleterious to specific stages of the vectors have been developed to suppress vector populations. Pathogens can be eliminated by genetically modified arthropods or commensal bacteria expressing effectors against the pathogen; by introducing a commensal, such as *Wolbachia*, that creates a physiological state nonpermissive to the pathogen; or by exposing the vector to drugs that target the pathogen, such as contact of mosquitoes with antimalarial drugs that eliminate *Plasmodium* parasites.

consequences for human and animal health. There is growing consensus that disrupting disease transmission by arthropod vectors is critical for disease eradication. As we continue to harness our growing understanding of the biology of pathogen transmission, we uncover critical interactions that can be targeted to prevent disease. New translational tools are constantly being developed that may allow us to witness the long-awaited eradication of some of the most devastating vector-borne diseases.

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