CellPress

Trends in Immunology

Opinion

Malaria: influence of *Anopheles* mosquito saliva on *Plasmodium* infection

Gunjan Arora [1], Yu-Min Chuang,[1] Photini Sinnis,[2] George Dimopoulos,[2] and Erol Fikrig[1,*]

Malaria is caused by *Plasmodium* protozoa that are transmitted by anopheline mosquitoes. *Plasmodium* sporozoites are released with saliva when an infected female mosquito takes a blood meal on a vertebrate host. Sporozoites deposited into the skin must enter a blood vessel to start their journey towards the liver. After migration out of the mosquito, sporozoites are associated with, or in proximity to, many components of vector saliva in the skin. Recent work has elucidated how *Anopheles* saliva, and components of saliva, can influence host–pathogen interactions during the early stage of *Plasmodium* infection in the skin. Here, we discuss how components of *Anopheles* saliva can modulate local host responses and affect *Plasmodium* infectivity. We hypothesize that therapeutic strategies targeting mosquito salivary proteins can play a role in controlling malaria and other vector-borne diseases.

Anopheles mosquitoes actively participate in the transmission of malaria

Malaria (see Glossary) caused >240 million clinical infections and >620 000 deaths worldwide in 2020; >90% of which occurred in sub-Saharan Africa [1]. *Plasmodium* sp., the causative agent of malaria, is transmitted by the bite of an infected female anopheline mosquito; arguably the most dangerous animal that humans encounter [2]. Mosquito-borne diseases are expanding their range, and the incidence of malaria is rising [3]. *Plasmodium* requires a mosquito and a human host to complete its lifecycle. The completion of parasite development in the mosquito's midgut results in the production of sporozoites, which migrate to, and invade, the *Anopheles* salivary glands. When a female mosquito probes for human blood, it deposits saliva and sporozoites into the skin. The importance of the salivary glands for harboring and transmitting *Plasmodium* remains unexplored. Of relevance, putative therapeutics targeting *Anopheles* salivary gland antigens might block parasite transmission and thus control malaria; in part, because sporozoites spend several hours at the inoculation site [4,5]. The success of *Plasmodium* infection depends on how well these few hundred sporozoites evade local immune responses before reaching blood vessels and migrating to the liver (Figure 1, Key figure) [6]. While most research efforts have focused on how *Plasmodium* antigens induce host responses, it is important to consider the tripartite molecular interactions between mosquito salivary proteins, *Plasmodium,* and the vertebrate host, following deposition of the sporozoites into the skin [7–10]. Recent work on salivary proteins such as anopheline antiplatelet protein (AAPP), AgTRIO, mosquito gamma-interferon-inducible lysosomal thiol reductase (mosGILT), sporozoite-associated mosquito saliva protein 1 (SAMSP1), and *Anopheles gambiae sporozoite-associated protein* (AgSAP) have revealed a new-found role for salivary proteins in regulating the immune cell function and modulating the infectivity of malaria parasites in the skin. In this opinion, we describe selected host responses to *Anopheles* bites and the role of individual salivary proteins in *Plasmodium* transmission. We hypothesize that targeting mosquito salivary proteins might help mitigate or control malaria disease outcomes, and therefore, should be further investigated.

Highlights

Mosquito saliva, which contains proteins with antihemostatic, anti-inflammatory, and immunomodulatory activities, is inoculated into the host during malaria parasite transmission.

Some saliva proteins associate with the *Plasmodium* sporozoite surface and may interfere in host–pathogen interactions.

Early work on a few *Anopheles* proteins indicates that specific saliva factors can negatively or positively affect *Plasmodium* transmission and infection.

Following a mosquito bite, components of its saliva remain in the skin for many hours and may alter the innate and adaptive immune response in the skin.

Some salivary proteins such as *Anopheles gambiae* sporozoite-associated protein (AgSAP), sporozoite-associated mosquito saliva protein 1 (SAMSP1), and AgTRIO can inhibit T cell responses and overall inflammatory reactions that may directly impact the survival of *Plasmodium* sporozoites.

Next-generation vaccines and monoclonal antibodies targeting salivary proteins may lead to the development of broad-spectrum therapeutics that can block the transmission of *Plasmodium* and other mosquito-borne pathogens.

Significance

Malaria begins when anopheline mosquitoes inject *Plasmodium* sporozoites, along with saliva when taking a blood meal, into the dermis of a vertebrate host. Mosquito saliva has pleiotropic effects that can influence local host immune responses. Although the role of mosquito saliva in malaria transmission has been speculated on for more than two decades, recent studies

 https://doi.org/10.1016/j.it.2023.02.005

Host responses to *Anopheles* bites

Mosquito bites lead to a local cutaneous reaction with swelling and erythema, sometimes followed by itching and the development of an **indurated papule**. Components of mosquito saliva remain in the human skin for up to 18 h, and the host response to these antigens contributes to inflammation. *Anopheles* bites lead to vasodilation, capillary extravasation, and hemorrhage, accompanied by infiltration of polymorphonuclear leukocytes and monocytes [11,12] (Figure 2). The cutaneous inflammatory response is partially dependent on tissue-resident mast cells, which are activated following mosquito bites [13], and saliva deposits that colocalize with degranulated mast cells [11]. In response to *Anopheles* bites, mast cells produce the chemokine CXCL-2 and enhance neutrophil recruitment in mice [13,14]. Moreover, impact of mosquito bites on mast cell response was further studied using–mouse models (WBB6F1-W/W^{v}) (mast-cell deficient) vs WBB6F1$^{+/+}$ (mast-cell sufficient). Mast cell activation led to the production of anti-inflammatory cytokine interleukin (IL)-10 and downregulation of antigen specific T cell responses (as evidenced from cytokine production) [13,14]. These studies suggested that *Anopheles* bites could have a differential impact on the activation of innate immunity and the inhibition of antigen-specific immune responses.

identified the role of salivary proteins in host–pathogen interactions. Elucidating the role of these proteins in modulating immune responses in the skin may help design new approaches to control the establishment of *Plasmodium* infection.

[1]Section of Infectious Diseases, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT, 06520, USA
[2]Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, USA

*Correspondence:
erol.fikrig@yale.edu (E. Fikrig).

Plasmodium sporozoites and associated salivary gland proteins influence the host response

Upon reaching the dermis, *Plasmodium* sporozoites navigate through the skin for many hours [9,15,16]. During this period, a fraction of the sporozoites invade the blood or lymphatic vessels while the sporozoites that do not successfully enter the blood stream remain in the dermis where they die and are cleared by host defenses [8,17]. Interactions between sporozoites and immune cells in the skin can influence the protective response to malaria [12,18–20]. This is attributed to the ability of sporozoites to migrate in the dermis and the immunosuppressive responses of *Plasmodium* or salivary proteins [20–22]. Specifically, saliva components from *Anopheles stephensi* inhibited *Plasmodium yoelii*-specific CD8^{+} T lymphocyte responses (in terms of T cell expansion frequencies) in sporozoite-primed mice, while purified sporozoites augmented T cell responses [23]. The inhibition of CD8^{+} T lymphocyte responses might be related to the mobilization of **regulatory T (Treg) cells** in the dermis following the bite of *Plasmodium*-infected mosquitoes [22]. Indeed, relative to controls, mosquito bite-induced Treg cells showed an increase in the expression of the homing molecule, L-selectin (CD62L), which is known to facilitate migration and homing of T cells to lymph nodes. The suppressive action on Th1 responses was also evident from an increase in IL-4 and IL-10 after inoculation of sporozoites in the skin [24]. While a majority of studies suggest that *Anopheles* salivary components suppress **T-helper 1 (Th1) responses**, another study reported that the exposure to an uninfected mosquito bite polarized the immune response towards a Th1 cell phenotype, as evidenced from the increased concentrations of IL-12, IFN-γ, and inducible nitric oxide synthase in the mouse liver [25]. These nonconcordant results are likely due to differences in the numbers of sporozoites inoculated and/or their route of inoculation, that is, via mosquito bite or intradermal inoculation of homogenized glands; however, further studies should clarify these discrepancies. We speculate that the mosquito bite suppresses Th1 responses in the skin and should be considered an important factor in the design of newer putative malaria vaccines.

Impact of *Anopheles* salivary gland proteins on *Plasmodium* transmission and infection

To develop a successful sporozoite-based vaccine, it is essential to understand the role of *Anopheles* saliva on the outcome of *Plasmodium* infection. Early studies with mice immunized with mosquito salivary gland homogenates showed modest protection against *Plasmodium berghei,* as evidenced from the changes in infectivity of mice injected with salivary gland extracts compared to control groups [26,27]. This was further substantiated by experiments in which the addition of salivary gland homogenates of *Aedes* mosquitoes increased *Plasmodium gallinaceum* infection in chickens when subcutaneously challenged; this showed that proteins present in

salivary glands could influence the outcomes of avian malaria parasite infection [28]. Furthermore, repeated pre-exposure to uninfected *Anopheles stephensi* has been proposed to evoke a Th1 response that contributes to some protection against *P. yoelii* infection [25]. The effect of mosquito bites on *Plasmodium* infection was also shown when mice were infected with *P. berghei* while concomitantly fed upon by *Anopheles*. Specifically, mice exposed to these mosquitoes exhibited increased progression to **cerebral malaria** with elevated IL-10 concentrations in the skin and draining lymph nodes compared to controls, suggesting that mosquito bites modulated the local immune environment in the skin to favor parasite survival [24]. Moreover, while these studies showed protective effects of mosquito salivary components, as evidenced by a lower *Plasmodium* burden following exposure to a mosquito bite or salivary components, one study showed that *A. stephensi* saliva did not influence infectivity of either *P. berghei* or *P. yoelii* [29] based on data from Giemsa-stained blood smears. However, in the latter Kebaier study [29], *Plasmodium* was detected at blood stage, 14 days postinfection, unlike the liver parasite burden measurements that were taken at 40 h postinfection in the Donovan study [25]; thus, the later study was unlikely able to detect the immediate impact of mosquito saliva. In a recent study, our group observed that antisera against *Anopheles gambiae* salivary glands, but not repeated mosquito bites, partially protected mice from mosquito-transmitted *Plasmodium* infection [30], as evidenced from the *Plasmodium* burden in mouse livers. This finding, together with individual studies on salivary gland antigens AgTRIO, SAMSP1, mosGILT, and AgSAP, suggest that *Anopheles* saliva has pleiotropic effects on host immunity, as well as on sporozoite mobility and infectivity [30–34].

Overall, these reports suggest that some saliva proteins, and the mosquito bite itself, have an influence on cutaneous host responses. As individuals living in sub-Saharan Africa may get thousands of bites each season, we hypothesize that the effect of these salivary proteins on skin immunity could be chronic and might play role in suppressing the immune response [35–38] in individuals living in malaria-endemic areas.

Components of *Anopheles* saliva

Proteomics

On finding the vertebrate host, an *Anopheles* mosquito searches for blood by piercing its proboscis into the host skin, which provides pathogens access to the subepithelial microenvironment. Insect saliva helps to optimize blood feeding and can influence capillary extravasation, hemostasis, pain and itch responses, and immune effector mechanisms [13,25,39–42]. To identify salivary proteins that are important in these processes and that also potentially alter *Plasmodium* transmission to the host, it is crucial to define the *Anopheles* salivary gland proteome. Different studies have identified up to 150 proteins present in *Anopheles* saliva and salivary glands. One study identified 69 unique proteins in the salivary gland of *A. gambiae* which include secretory molecules, housekeeping genes, and proteins of unknown function [43]. The study uncovered the D7r family proteins, apyrases, and the salivary gland-like (SG-like) family, as well as mosGILT, AgTRIO, and SAMSP1 homologs in the salivary glands of *A. gambiae* [43]. Another proteomics study characterized five proteins in saliva and 122 proteins in the salivary glands of *A. gambiae* [40]. Using quantitative proteomics, the study showed that the expression of a defense-related protein gVAG was increased twofold in the infected salivary glands, whereas expression of four proteins was decreased – gSG6, apyrase, D7 related-1 protein precursor, and D7 precursor allergen AED A2, compared with uninfected *Anopheles* mosquitoes [44]. Overall, these and other proteomics studies suggest that *Anopheles* saliva is dynamic and complex, and that *Plasmodium* infection can modulate the expression of components of saliva. From these studies, it is clear that *Anopheles* not only provides malaria parasites the physical access to invade the host but also express individual saliva proteins that help *Plasmodium* to reach the next stage of its lifecycle [45–48].

Glossary

Cerebral malaria: one of the most serious malaria complications; characterized by coma and neurological manifestations.
Circumsporozoite protein (CSP): the major surface protein of the *Plasmodium* sporozoite; major vaccine target. CSP helps *Plasmodium* sporozoite adhesion to target cells.
Gamma-interferon-inducible lysosomal thiol reductase (GILT): a thioredoxin-related oxidoreductase; functions by reducing disulfide bonds of endocytosed proteins in antigen-presenting cells.
Indurated papule: small, solid, raised, and hardened bump on the skin.
Malaria: serious and sometimes fatal disease caused by a *Plasmodium* parasite that is transmitted by the bite of an infected female *Anopheles* mosquito.
Prediuresis: process by which blood-feeding mosquitoes excrete drops of fluid to concentrate ingested blood protein within a small volume that fits in their midgut.
Regulatory T (Treg) cells: subpopulation of T cells that maintains homeostasis and self-tolerance in the body. Treg cells suppress the action of other immune events to keep the immune system from becoming overactive.
Sialomes: proteins expressed in the salivary glands of mosquitoes and other blood-feeding arthropods.
T helper type 1 (Th1) responses: Th1 cell response maintains cell-mediated immune responses and produces proinflammatory cytokines such as IFN-γ.
Yeast surface display: protein engineering technique that expresses recombinant proteins on the surface of yeast and is used to find novel host–vector–pathogen interactions.

Key Figure

Role of *Anopheles* saliva antigens in blood feeding

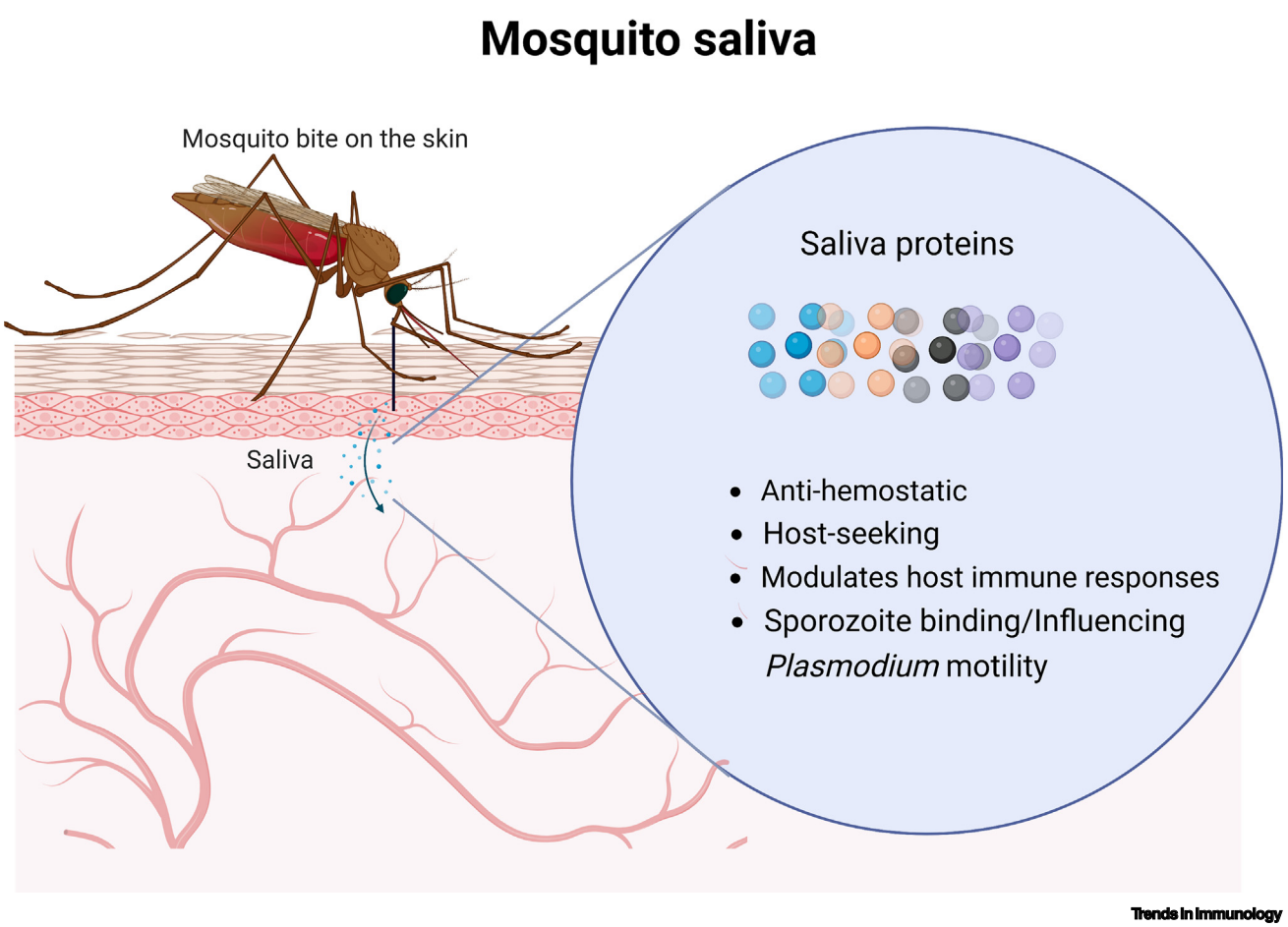

Figure 1. This figure was created with Biorender.com.

Sporozoite-associated salivary proteins

Upon reaching the skin, sporozoites may be coated with secretory or basement membrane proteins from *Anopheles* salivary glands via specific interactions or charge-based electrostatic interactions, shown via molecular and cellular studies [33,34]. To identify sporozoite-associated mosquito proteins, the saliva of infected *A. gambiae* were subjected to mass spectrometry [33]. The screening led to the identification of mosGILT as a major sporozoite-associated protein [33]. mosGILT binds to both *P. berghei* and *P. falciparum*, and homologs are present in other species of *Anopheles* [33]. The screen also led to the identification and characterization of SAMSP1 and AgSAP as additional proteins that associate with *Plasmodium* sporozoites, as well as other potential targets [33]. This type of analysis provides a focus on proteins that may interact with the sporozoite surface and thereby influence host–pathogen interactions. However, more work is needed to elucidate how *Plasmodium* interactions with mosquito salivary glands may affect sporozoite infectivity and transmission to the host.

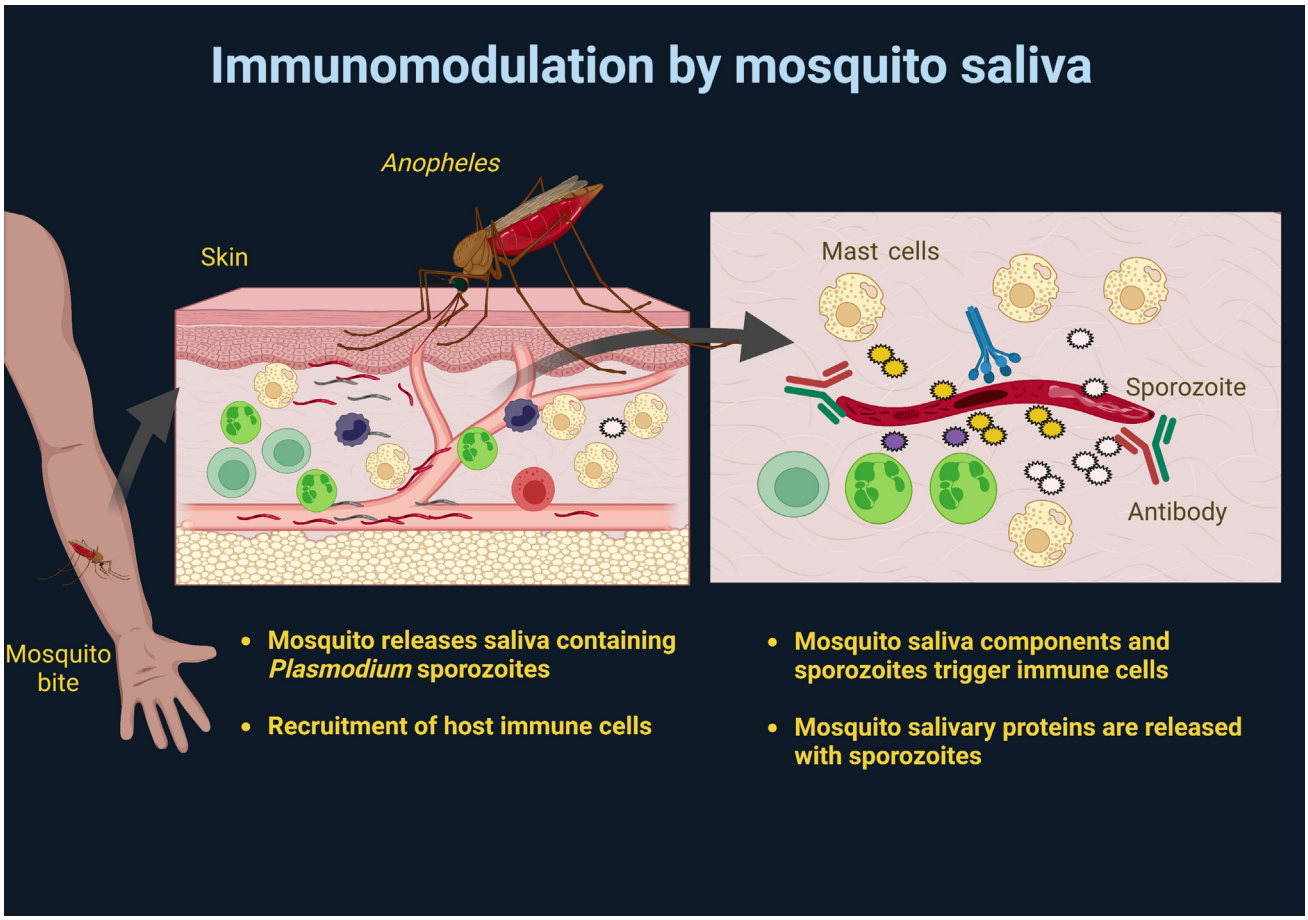

Figure 2. Mosquito bite impact on host immunity. Saliva antigens influence host immunity and *Plasmodium sp.* infection. This figure was created with Biorender.com.

Role of individual salivary gland proteins in blood feeding or *Plasmodium* infection

Anopheline antiplatelet protein

AAPP is a protein that was first identified in the saliva of *A. stephensi* [49]. AAPP binding to collagen can inhibit thrombosis since it interferes with platelet adhesion to collagen [49]. Furthermore, *in vitro* results show that AAPP binding to collagen I and III limits the ability of immunoglobulin-like receptor glycoprotein VI, present on the platelet surface, to bind to collagen [49]. AAPP also inhibits the collagen-mediated increase in intracellular Ca^{2+} concentration in platelets [49]. This is relevant because the platelets trigger the mechanical pathway of the coagulation cascade by binding to collagen and stop blood leakage. AAPP inhibits platelet function and allows the mosquito to feed on the host. The ability of AAPP to inhibit blood aggregation suggests potential clinical utility as an anticoagulant [50]. Moreover, the neutralization of AAPP in transgenic *A. stephensi* mosquitoes also affects factors associated with blood feeding, including probing and **prediuresis** time, blood meal size, and fecundity [51]. The fitness of mosquitoes has been affected when a transgenesis-based protein inactivation approach was used to express anti-AAPP antibody single-chain fragment (scFv) in the salivary glands of *A. stephensi*. AAPP neutralization did not affect oocyst numbers in mosquitoes when fed upon *P. berghei*-infected

mice. Overall, these studies demonstrate a role for AAPP in blood feeding and as a platelet-modulating agent, certainly meriting further attention.

AgTRIO

Since mosquito saliva contains hundreds of proteins, a **yeast surface display** approach was used to identify proteins that react with serum from rabbits immunized with a salivary gland extract [30]. Twenty-one *A. gambiae* proteins were uncovered, including AgTRIO, D7r1, and six other antigens with putative signal sequences. AgTRIO is part of an *Anopheles*-specific SG1 gene family [52] and AgTRIO expression is increased by the presence of *P. berghei* in the salivary glands of mosquitoes [53,54]. AgTRIO antisera reduced the pathogen titers in mice bitten by mosquitoes infected with *P. berghei* or *P. falciparum*, and synergized with *Plasmodium* **circumsporozoite protein (CSP)** antibodies for enhanced efficacy [30]. To our knowledge, AgTRIO was the first example in which neutralizing a mosquito protein but not a parasite protein provided some degree of protection. Moreover, antibodies targeting the carboxyl terminus of AgTRIO were associated with partial immunity [76]. While the immune mechanism by which AgTRIO provides protection remains unclear, a study showed that AgTRIO mouse antiserum decreased sporozoite movement in the dermis, and that AgTRIO inhibited the expression of tumor necrosis factor (TNF)-α in mouse skin [32]. Overall, to our knowledge, AgTRIO might be the first example of an *Anopheles* salivary protein facilitating *Plasmodium* infection by influencing the local environment in the vertebrate host [30,32].

mosGILT

It is also possible that some mosquito salivary gland proteins may negatively impact *Plasmodium* during the movement of sporozoite to the vertebrate host. One such protein is mosGILT. Specifically, mosGILT was identified by a mass spectrometry screen of saliva from *A. gambiae* infected with *P. berghei* [33]. mosGILT shares homology with human GILT, an immune-related protein involved in antigen processing and presentation [55]. mosGILT binds to *Plasmodium* and impacts sporozoite cell traversal activity, an important process used by sporozoites as they migrate through the dermis and towards the blood vessels and liver of the host [33]. Intravital imaging in mice showed that mosGILT reduced the speed of *Plasmodium* sporozoites in the skin but did not affect *Plasmodium* viability [33]. In addition, mosGILT binding to sporozoites reduced *Plasmodium* infection and, conversely, mice immunized with mosGILT showed higher parasite burdens in the liver than ovalbumin-immunized mice, suggesting that mosGILT could modulate the level of initial *Plasmodium* infection in mice [33]. Overall, the interaction between mosGILT and *Plasmodium* sporozoites may suggest an unknown mechanism by which sporozoite motility is regulated in salivary glands; this may be interesting to evaluate because such motility might influence the ability of *Plasmodium* to be released from mosquito salivary glands.

In addition to influencing *Plasmodium* transmission, mosGILT, is essential for mosquito reproduction and protects *Plasmodium* oocysts from destruction within the mosquito [56]. This shows that some *Anopheles* proteins may have multiple functions, including *Anopheles* development, innate immunity, and control of *Plasmodium* infection. Therefore, it is important to study the collective role of such proteins both in *Anopheles* biology and malaria immune responses.

SAMSP1

SAMSP1 is another mosquito protein that interacts with *Plasmodium* during migration from the vector to the vertebrate host. In contrast to mosGILT, cellular assays showed that SAMSP1 binding to *P. berghei* enhanced sporozoite gliding and traversal [31]. SAMSP1

also affects host immunity at the bite site. Specifically, mouse intradermal inoculation of *Anopheles* salivary gland extracts combined with SAMSP1 decreased the number of neutrophils at the inoculation site compared with controls [31]. The influence of SAMSP1 on skin immunity was associated with neutrophil recruitment since there were no effects on macrophage, Langerhans cell, or dendritic cell numbers compared with controls. This was also evident in an *in vitro* assay in which SAMSP1 altered neutrophil chemotaxis. Neutralization of SAMSP1 by active immunization or by passive transfer of antibodies diminished the *Plasmodium* burden in the liver of mice relative to mice that received ovalbumin antisera [31]. Overall, these findings suggest that SAMSP1 helps facilitate malaria infection. Furthermore, when mice were intravenously given antibodies against both SAMSP1 and CSP, the *Plasmodium* burden decreased more than when CSP antibodies were given alone [31]. Consistent with this, monoclonal antibodies against CSP are currently being tested in clinical trials to prevent malaria, and we argue that the addition of AgTRIO, SAMSP1, or other *Anopheles* salivary gland protein antibodies to CSP, might potentially enhance the efficacy of these antibodies.

AgSAP

Combining proteomic analysis of saliva with sporozoite-associated proteins has provided additional information on targets that were previously not described in **sialomes**. For example, mass spectrometry of sporozoites isolated from *Anopheles* saliva led to the identification of a protein named *A. gambiae* sporozoite-associated protein (AgSAP) that is not a secretory protein. However, it associates with *P. falciparum* and *P. berghei* sporozoites, as evidenced from proteomics studies and visualization using specific antibodies [33,34]. AgSAP has some homology to the *Drosophila* protein Papilin, an extracellular matrix protein that inhibits metalloproteinases [33,57]. AgSAP binds to heparan sulfate (HS), a glycosaminoglycan present on the surface of mammalian cells and in the extracellular matrix [34,58–60]. HS moieties interact with various proteins including selectins (shown by *in vitro* studies with monocyte–endothelial interaction models and by *in vivo* mouse studies with endothelial cells); they play an important role in inflammation [61–65]. For instance, AgSAP–HS interactions can interfere with multiple steps of inflammation: AgSAP modulates local inflammatory response in mouse skin and inhibits expression of TNF-α and various other proinflammatory cytokines such as IL-1β, IFN-γ, IL-4, matrix metalloproteinase-9, transforming growth factor-β, and ligands such as intercellular adhesion molecule-1 [34]. AgSAP inhibits activation of human Jurkat T cells *in vitro*, and *AgSAP* silencing via RNAi in *Anopheles* mosquitoes also reduces effective transmission of sporozoites to mice [34]. These studies suggest that AgSAP might help sporozoites suppress immune responses in the skin and facilitate *Plasmodium* infection in the vertebrate host, although further investigations are warranted.

Apyrase

Plasmodium infection influences mosquito behavior, including the ability of the mosquito to bite more frequently [66,67]. Sporozoites inhibit the activity of apyrase, a salivary protein that hydrolyzes ADP and inhibits ADP-induced platelet aggregation. Studies have shown that salivary apyrase concentrations are inversely correlated with probing time, suggesting that *Plasmodium* infection alters the composition of *Anopheles* saliva to make mosquitoes more likely to feed again, and more persistent in their host-seeking behavior. [66,67]. Although intriguing, the influence of apyrase on *Plasmodium* transmission remains unknown.

D7 protein family

D7 proteins are among the most abundant in *Anopheles* saliva and were initially characterized as odorant binding proteins [68]. *A. gambiae*, the primary vector of malaria, expresses multiple long-

and short-form D7 proteins [69]. A recent study showed that *A. gambiae* D7 long-forms bind to hemostatic agonists such as leukotriene C4 and serotonin (potent activators of vasoconstriction, edema, and postcapillary venule leakage) [69]. AngaD7L1 also binds and inhibits platelet aggregation and the vasoconstrictive activity of thromboxane A2 analog U-46619 [69]. In addition, AngaD7L3 inhibits serotonin-induced platelet aggregation and vasoconstriction [69]. AngaD7L proteins counteract host hemostasis by interacting with factors XII, XIIa, and XI in blood, and show a dose-dependent anticoagulant effect *in vitro* [69]. Our group previously showed that neutralizing D7r1 with specific rabbit antiserum did not alter the *Plasmodium* load in the liver, as evidenced by quantitative PCR; however, the role of other D7 proteins remains to be investigated [30]. Overall, these studies suggest that D7 might play a role in blood feeding.

Concluding remarks

Human populations in sub-Saharan Africa and other malaria endemic areas are continuously exposed to mosquitoes during the transmission season. In these regions, mosquito saliva components and their influence on adaptive immune responses may have an impact on shaping natural immunity to malaria [70–73]. In addition to interfering with host–*Plasmodium* interactions, mosquito salivary gland proteins may alter sporozoite infectivity. Indeed, as reviewed here, recent work on a few *Anopheles* proteins suggests that specific saliva factors can have a negative, or positive, effect on *Plasmodium* transmission and infection. The role of most saliva proteins remains unknown and based on limited studies performed by different groups, we hypothesize that *Anopheles* saliva may not only help in blood feeding processes, but also actively participate in *Plasmodium* transmission to the host (see Outstanding questions). The examples described in this opinion indicate that detailed studies are needed to decipher the effect of *Anopheles* proteins on *Plasmodium* in the arthropod and vertebrate hosts [33,56] (Figure 1). These issues are relevant as humans living in malaria-endemic areas are exposed to both uninfected and infected mosquitoes and therefore, it is difficult to infer from longitudinal studies in malaria-endemic areas, the nature of the antibody responses to *Anopheles* salivary proteins that are related to *Plasmodium* infection.

A vaccine that might block pathogen transmission and restrict mosquitoes from harboring or transmitting malaria parasites is one of the challenging goals of research on arthropod-borne diseases. The field of vector-targeted vaccines is in its infancy but recently an mRNA-based vaccine targeting 19 tick proteins (19ISP) provided protection from infection with the Lyme disease agent, *Borrelia burgdorferi* [74]. The ability of the ticks to feed on guinea pigs immunized with 19ISP mRNA-LNP cocktail was substantially reduced when compared with guinea pigs immunized with a nonspecific mRNA-LNP. Quantitative PCR showed that the majority of the 19ISP-vaccinated guinea pigs remained uninfected compared to the control group in which most animals were infected with *B. burgdorferi* [74]. Another advance is the development of a mosquito cocktail containing four *A. gambiae* saliva antigen peptides, but more work is needed to determine whether this cocktail provides any protection against malaria [75]. Unlike *Ixodes* ticks for which the 19ISP mRNA vaccine induces erythema and restricts the ability of the arthropods to feed on the host [74], a mosquito saliva-targeted vaccine has to work in a different manner because mosquitoes feed rapidly and individuals living in endemic areas are exposed to hundreds of mosquito bites every day. More immunological and functional studies are needed to understand the function of specific saliva proteins and their role in *Plasmodium* infectivity during the initial stages of infection in the vertebrate host. Nevertheless. it is essential to study *Anopheles* salivary proteins since some of these proteins coat *Plasmodium* sporozoites, and may create an immunoprivileged niche at the bite site that hinders immune cells from eliminating sporozoites in the skin. Therefore, we posit that adding an *Anopheles*-based component to current *Plasmodium*-based vaccines might represent a paradigm shift in malaria control and a means to enhance protection against different species of *Plasmodium.*

Outstanding questions

How does a mosquito bite regulate the inflammatory response in the skin? What are the unique immunological features of the *Anopheles* bite compared to other mosquitoes and other arthropods such as ticks?

How does *Plasmodium* sporozoite trafficking to the mosquito salivary glands impact the secretion of certain salivary proteins, which in turn influence sporozoite infectivity?

What host receptor interacts with salivary proteins such as Apyrase, AgTRIO, SAMSP1, and AgSAP?

Which salivary proteins might serve as putative biomarkers for epidemiological analysis of malarial transmission?

What role does the skin microbiota plays in mosquito bites and *Plasmodium* infection?

Could a cocktail of *Anopheles* salivary antigens – stand-alone or in combination with *Plasmodium* antigens – be used as an effective candidate anti-malarial vaccine?

Acknowledgments

This research was supported in part by the Howard Hughes Medical Institute Emerging Pathogens Initiative and National Institutes of Health grant AI158615.

Declaration of interests

No interests are declared.

References

1. World Health Organization (2021) *World Malaria Report 2021*, World Health Organization, Geneva
2. Crompton, P.D. *et al.* (2014) Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. *Annu. Rev. Immunol.* 32, 157–187
3. Colon-Gonzalez, F.J. *et al.* (2021) Projecting the risk of mosquito-borne diseases in a warmer and more populated world: a multi-model, multi-scenario intercomparison modelling study. *Lancet Planet Health* 5, e404–e414
4. Hopp, C.S. *et al.* (2021) Comparative intravital imaging of human and rodent malaria sporozoites reveals the skin is not a species-specific barrier. *EMBO Mol. Med.* 13, e11796
5. Yamauchi, L.M. *et al.* (2007) Plasmodium sporozoites trickle out of the injection site. *Cell. Microbiol.* 9, 1215–1222
6. Hopp, C.S. and Sinnis, P. (2015) The innate and adaptive response to mosquito saliva and Plasmodium sporozoites in the skin. *Ann. N. Y. Acad. Sci.* 1342, 37–43
7. Kehrer, J. *et al.* (2022) *Plasmodium* sporozoite disintegration during skin passage limits malaria parasite transmission. *EMBO Rep.* 23, e54719
8. Flores-Garcia, Y. *et al.* (2018) Antibody-mediated protection against Plasmodium sporozoites begins at the dermal inoculation site. *mBio* 9, e02194-18
9. Hopp, C.S. *et al.* (2015) Longitudinal analysis of *Plasmodium* sporozoite motility in the dermis reveals component of blood vessel recognition. *Elife* 4, e07789
10. Menard, R. *et al.* (2013) Looking under the skin: the first steps in malarial infection and immunity. *Nat. Rev. Microbiol.* 11, 701–712
11. Choumet, V. *et al.* (2012) Visualizing non infectious and infectious *Anopheles gambiae* blood feedings in naive and saliva-immunized mice. *PLoS One* 7, e50464
12. Mac-Daniel, L. *et al.* (2014) Local immune response to injection of *Plasmodium* sporozoites into the skin. *J. Immunol.* 193, 1246–1257
13. Demeure, C.E. *et al.* (2005) Anopheles mosquito bites activate cutaneous mast cells leading to a local inflammatory response and lymph node hyperplasia. *J. Immunol.* 174, 3932–3940
14. Depinay, N. *et al.* (2006) Mast cell-dependent down-regulation of antigen-specific immune responses by mosquito bites. *J. Immunol.* 176, 4141–4146
15. Amino, R. *et al.* (2006) Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. *Nat. Med.* 12, 220–224
16. Vaughan, J.A. *et al.* (1999) Infectivity of *Plasmodium berghei* sporozoites delivered by intravenous inoculation versus mosquito bite: implications for sporozoite vaccine trials. *Infect. Immun.* 67, 4285–4289
17. Aliprandini, E. *et al.* (2018) Cytotoxic anti-circumsporozoite antibodies target malaria sporozoites in the host skin. *Nat. Microbiol.* 3, 1224–1233
18. Haeberlein, S. *et al.* (2017) Protective immunity differs between routes of administration of attenuated malaria parasites independent of parasite liver load. *Sci. Rep.* 7, 10372
19. Nganou-Makamdop, K. *et al.* (2012) Reduced *Plasmodium berghei* sporozoite liver load associates with low protective efficacy after intradermal immunization. *Parasite Immunol.* 34, 562–569
20. Winkel, B.M.F. *et al.* (2020) *Plasmodium* sporozoites induce regulatory macrophages. *PLoS Pathog.* 16, e1008799
21. de Korne, C.M. *et al.* (2021) Clustering and erratic movement patterns of syringe-injected versus mosquito-inoculated malaria sporozoites underlie decreased infectivity. *mSphere* 6, e00218-21
22. da Silva, H.B. *et al.* (2012) Early skin immunological disturbance after *Plasmodium*-infected mosquito bites. *Cell. Immunol.* 277, 22–32
23. Billman, Z.P. *et al.* (2016) Purification of *Plasmodium* sporozoites enhances parasite-specific CD8+ T cell responses. *Infect. Immun.* 84, 2233–2242
24. Schneider, B.S. *et al.* (2011) *Anopheles stephensi* saliva enhances progression of cerebral malaria in a murine model. *Vector Borne Zoonotic Dis.* 11, 423–432
25. Donovan, M.J. *et al.* (2007) Uninfected mosquito bites confer protection against infection with malaria parasites. *Infect. Immun.* 75, 2523–2530
26. Alger, N.E. and Harant, J. (1976) *Plasmodium berghei*: sporozoite challenge, protection, and hypersensitivity in mice. *Exp. Parasitol.* 40, 273–280
27. Alger, N.E. *et al.* (1972) Sporozoite and normal salivary gland induced immunity in malaria. *Nature* 238, 341
28. Rocha, A.C. *et al.* (2004) Effect of the *Aedes fluviatilis* saliva on the development of *Plasmodium gallinaceum* infection in *Gallus* (*gallus*) *domesticus*. *Mem. Inst. Oswaldo Cruz* 99, 709–715
29. Kebaier, C. *et al.* (2010) Neither mosquito saliva nor immunity to saliva has a detectable effect on the infectivity of *Plasmodium* sporozoites injected into mice. *Infect. Immun.* 78, 545–551
30. Dragovic, S.M. *et al.* (2018) Immunization with AgTRIO, a protein in Anopheles saliva, contributes to protection against *Plasmodium* infection in mice. *Cell Host Microbe* 23, 523–535 e5
31. Chuang, Y.M. *et al.* (2021) The effects of a mosquito salivary protein on sporozoite traversal of host cells. *J. Infect. Dis.* 224, 544–553
32. Chuang, Y.M. *et al.* (2019) *Anopheles gambiae* lacking AgTRIO inefficiently transmits *Plasmodium berghei* to mice. *Infect. Immun.* 87, e00326-19
33. Schleicher, T.R. *et al.* (2018) A mosquito salivary gland protein partially inhibits *Plasmodium* sporozoite cell traversal and transmission. *Nat. Commun.* 9, 2908
34. Arora, G. *et al.* (2021) Immunomodulation by mosquito salivary protein AgSAP contributes to early host infection by *Plasmodium*. *mBio* 12, e0309121
35. Guha, R. *et al.* (2021) *Plasmodium falciparum* malaria drives epigenetic reprogramming of human monocytes toward a regulatory phenotype. *PLoS Pathog.* 17, e1009430
36. Farrington, L. *et al.* (2017) Both inflammatory and regulatory cytokine responses to malaria are blunted with increasing age in highly exposed children. *Malar. J.* 16, 499
37. Greenwood, B.M. *et al.* (1972) Immunosuppression in children with malaria. *Lancet* 1, 169–172
38. Portugal, S. *et al.* (2015) Malaria-associated atypical memory B cells exhibit markedly reduced B cell receptor signaling and effector function. *Elife* 4, e07218
39. Lefteri, D.A. *et al.* (2022) Mosquito saliva enhances virus infection through sialokinin-dependent vascular leakage. *Proc. Natl. Acad. Sci. U. S. A.* 119, e2114309119
40. Hastings, A.K. *et al.* (2019) *Aedes aegypti* NeSt1 protein enhances Zika virus pathogenesis by activating neutrophils. *J. Virol.* 93, e00395-19
41. Pingen, M. *et al.* (2017) Mosquito biting modulates skin response to virus infection. *Trends Parasitol.* 33, 645–657
42. Marin-Lopez, A. *et al.* (2021) AgBR1 and NeSt1 antisera protect mice from *Aedes aegypti*-borne Zika infection. *Vaccine* 39, 1675–1679
43. Kalume, D.E. *et al.* (2005) A proteomic analysis of salivary glands of female *Anopheles gambiae* mosquito. *Proteomics* 5, 3765–3777
44. Choumet, V. *et al.* (2007) The salivary glands and saliva of *Anopheles gambiae* as an essential step in the *Plasmodium* life cycle: a global proteomic study. *Proteomics* 7, 3384–3394

45. Rawal, R. *et al.* (2016) Towards a proteomic catalogue and differential annotation of salivary gland proteins in blood fed malaria vector *Anopheles culicifacies* by mass spectrometry. *PLoS One* 11, e0161870
46. Vijay, S. *et al.* (2014) Mass spectrometry based proteomic analysis of salivary glands of urban malaria vector *Anopheles stephensi*. *Biomed. Res. Int.* 2014, 686319
47. Fontaine, A. *et al.* (2012) Anopheles salivary gland proteomes from major malaria vectors. *BMC Genomics* 13, 614
48. Chaerkady, R. *et al.* (2011) A proteogenomic analysis of *Anopheles gambiae* using high-resolution Fourier transform mass spectrometry. *Genome Res.* 21, 1872–1881
49. Yoshida, S. *et al.* (2008) Inhibition of collagen-induced platelet aggregation by anopheline antiplatelet protein, a saliva protein from a malaria vector mosquito. *Blood* 111, 2007–2014
50. Hayashi, H. *et al.* (2012) Anopheline anti-platelet protein from a malaria vector mosquito has anti-thrombotic effects *in vivo* without compromising hemostasis. *Thromb. Res.* 129, 169–175
51. Islam, A. *et al.* (2019) Anopheline antiplatelet protein from mosquito saliva regulates blood feeding behavior. *Sci. Rep.* 9, 3129
52. Arca, B. *et al.* (2005) An updated catalogue of salivary gland transcripts in the adult female mosquito, *Anopheles gambiae*. *J. Exp. Biol.* 208, 3971–3986
53. Marie, A. *et al.* (2014) *Anopheles gambiae* salivary protein expression modulated by wild *Plasmodium falciparum* infection: highlighting of new antigenic peptides as candidates of *An. gambiae* bites. *Parasit. Vectors* 7, 599
54. Zocevic, A. *et al.* (2013) New markers in *Anopheles gambiae* salivary glands after *Plasmodium berghei* infection. *Vector Borne Zoonotic Dis.* 13, 119–127
55. Maric, M. *et al.* (2001) Defective antigen processing in GILT-free mice. *Science* 294, 1361–1365
56. Yang, J. *et al.* (2020) Disruption of mosGILT in Anopheles gambiae impairs ovarian development and *Plasmodium* infection. *J. Exp. Med.* 217, e20190682
57. Kramerova, I.A. *et al.* (2000) Papilin in development; a pericellular protein with a homology to the ADAMTS metalloproteinases. *Development* 127, 5475–5485
58. Belting, M. (2003) Heparan sulfate proteoglycan as a plasma membrane carrier. *Trends Biochem. Sci.* 28, 145–151
59. Heremans, A. *et al.* (1988) Heparan sulfate proteoglycan from the extracellular matrix of human lung fibroblasts. Isolation, purification, and core protein characterization. *J. Biol. Chem.* 263, 4731–4739
60. Carey, D.J. and Todd, M.S. (1986) A cytoskeleton-associated plasma membrane heparan sulfate proteoglycan in Schwann cells. *J. Biol. Chem.* 261, 7518–7525
61. Collins, L.E. and Troeberg, L. (2019) Heparan sulfate as a regulator of inflammation and immunity. *J. Leukoc. Biol.* 105, 81–92
62. Farrugia, B.L. *et al.* (2018) The role of heparan sulfate in inflammation, and the development of biomimetics as anti-inflammatory strategies. *J. Histochem. Cytochem.* 66, 321–336
63. Parish, C.R. (2006) The role of heparan sulphate in inflammation. *Nat. Rev. Immunol.* 6, 633–643
64. Wang, L. *et al.* (2005) Endothelial heparan sulfate deficiency impairs L-selectin- and chemokine-mediated neutrophil trafficking during inflammatory responses. *Nat. Immunol.* 6, 902–910
65. Giuffre, L. *et al.* (1997) Monocyte adhesion to activated aortic endothelium: role of L-selectin and heparan sulfate proteoglycans. *J. Cell Biol.* 136, 945–956
66. Thievent, K. *et al.* (2019) Malaria load affects the activity of mosquito salivary apyrase. *J. Insect Physiol.* 116, 10–16
67. Rossignol, P.A. *et al.* (1984) Increased intradermal probing time in sporozoite-infected mosquitoes. *Am. J. Trop. Med. Hyg.* 33, 17–20
68. Calvo, E. *et al.* (2006) Function and evolution of a mosquito salivary protein family. *J. Biol. Chem.* 281, 1935–1942
69. Smith, L.B. *et al.* (2022) Novel salivary antihemostatic activities of long-form D7 proteins from the malaria vector *Anopheles gambiae* facilitate hematophagy. *J. Biol. Chem.* 298, 101971
70. Aka, K.G. *et al.* (2020) Pattern of antibody responses to *Plasmodium falciparum* antigens in individuals differentially exposed to *Anopheles* bites. *Malar. J.* 19, 83
71. Rochford, R. and Kazura, J. (2020) Introduction: immunity to malaria. *Immunol. Rev.* 293, 5–7
72. Sarr, J.B. *et al.* (2012) Differential acquisition of human antibody responses to *Plasmodium falciparum* according to intensity of exposure to *Anopheles* bites. *Trans. R. Soc. Trop. Med. Hyg.* 106, 460–467
73. Sarr, J.B. *et al.* (2007) Evaluation of antibody response to *Plasmodium falciparum* in children according to exposure of *Anopheles gambiae* s.l or *Anopheles funestus* vectors. *Malar. J.* 6, 117
74. Sajid, A. *et al.* (2021) mRNA vaccination induces tick resistance and prevents transmission of the Lyme disease agent. *Sci. Transl. Med.* 13, eabj9827
75. Manning, J.E. *et al.* (2020) Safety and immunogenicity of a mosquito saliva peptide-based vaccine: a randomised, placebo-controlled, double-blind, phase 1 trial. *Lancet* 395, 1998–2007
76. Chuang, Y.M. *et al.* (2022) A mosquito AgTRIO monoclonal antibody reduces early plasmodium infection of mice. *Infect. Immun.* 90, e0035921