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Flipping the paradigm on malaria transmission-blocking vaccines

Rhoel R. Dinglasan and Marcelo Jacobs-Lorena

Johns Hopkins Malaria Research Institute, Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, 615 N. Wolfe Street, Baltimore, MD 21205, USA

Abstract

The idea of malaria transmission-blocking vaccines (TBVs) surfaced more than two decades ago. Since then, the research paradigm focused on developing TBVs that target surface antigens of parasite sexual stages. Only recently has an effort emerged that flipped this paradigm, targeting antigens of the parasite's obligate invertebrate vector, the *Anopheles* mosquito. Here, we review the current state of knowledge of mosquito-based TBVs and discuss the utility of this approach for future vaccine development.

Preventing malaria by blocking parasite transmission

Successful production of malaria vaccines has been hindered by the same obstacles inherent in the development of any vaccine (reviewed in Ref. [1]). However, in addition to the RTS,S liver-stage vaccine [2] (Figure 1; see Glossary), the malaria transmission-blocking vaccine (TBV) approach has also achieved significant gains over the past decade, despite its real and perceived shortcomings.

Plasmodium parasites undergo an obligatory developmental cycle in the mosquito, and, thus, mosquitoes are responsible for the spread of the malaria parasite. As their name suggests, TBVs target the parasite stages (Box 1) that develop within the mosquito vector and, as a result, prevent the subsequent cascade of events that leads to infection of human hosts. All malaria vaccine approaches (Box 1) are intended to introduce herd immunity in the target community. Unlike classical vaccine approaches, TBVs do not protect the vaccinated individual from contracting malaria but are intended to prevent parasite development in the mosquito and thereby limit the number of infectious vectors. Consequently, TBVs have been categorized as 'altruistic vaccines' and undervalued (Box 2). Two main paradigms can be used in the design of TBVs: they could target either parasite or mosquito antigens. A comprehensive review of sexual- or sporogonic-stage TBVs has been published recently [20] (Box 1), and, thus, this area will not be covered in detail here. This review focuses on TBVs that target mosquito antigens. The general idea of all TBVs is that inhibitory antibodies against parasite or mosquito midgut antigens are taken up into the mosquito

midgut during blood feeding and, therefore, the inhibitory capacity of TBVs is highly dependent on eliciting a strong humoral response in vaccinated individuals. However, the potential benefit of inducing, in parallel, a host cellular immune response that reduces vector competence cannot be ruled out.

Box 1

Stages of parasite development

Liver-stage development

Sporozoites are inoculated into the bloodstream of the vertebrate host by the bite of an infected anopheline mosquito. Within minutes, sporozoites home in and infect the liver, where they develop into exoerythrocytic forms. These so-called ‘liver-stage parasites’ develop within the hepatocyte into a hepatic schizont. Merozoites that are formed from the schizonts are released into the bloodstream to begin the asexual blood-stage cycle.

Asexual blood-stage development

Hepatic merozoites invade red blood cells to form ring stages, the first of the erythrocytic- or blood-stage forms. Each ring stage further develops into trophozoites that divide to form schizonts. Schizonts mature into merozoites that are released from the red blood cells and the cycle is repeated when merozoites invade another red blood cell. The entire cycle for *P. falciparum* takes ~48 h.

Sexual mosquito-stage development

In the vertebrate blood stream, a small proportion of the parasites develop into sexual stages, called ‘gametocytes’. Gametocytes are the only form that can develop in the mosquito. Once ingested by the mosquito, gametocytes differentiate into male and female gametes that fertilize to form a zygote and then the motile form, the ookinete. Ookinetes move in the bloodmeal until they encounter and invade the midgut epithelium. After emerging on the basal side, facing the hemocoel, the ookinete transforms into an oocyst within which thousands of sporozoites develop. Once the oocyst ruptures ~10 days later, the sporozoites are released into the hemocoel, where they circulate and specifically recognize and invade the salivary glands. When the mosquito takes another bloodmeal the sporozoites are released with the saliva into the next vertebrate host.

Box 2

Why altruism is a good thing

TBVs set off a bottleneck by drastically reducing the circulating parasite population. TBVs have been dubbed ‘altruistic vaccines’ because they are intended to induce herd immunity in the population without directly protecting the vaccinated individual. This description tends to cause a negative perspective on the utility of these vaccines, but the following potential advantages should be noted:

- i. TBVs could limit the progression of malaria epidemics in areas of unstable malaria transmission.

- ii. TBVs could reduce the spread of multidrug-resistant parasites and effectively prolong the efficacious use of existing drugs.
- iii. When combined with liver-, blood- and sexual-stage vaccines, mosquito-based TBVs could prevent transmission of escape mutants (i.e. parasites that have developed resistance to the other types of vaccines).

Although *Plasmodium falciparum* is the deadliest of the four human malaria parasite species (Box 3), *Plasmodium vivax* also remains a significant global burden on human health [3]. Moreover, a recent report suggests that non-human primate malaria parasites also can be naturally transmitted into human populations [4]. Thus, production of parasite-based TBVs that are reactive with each species is essential. For example, separate groups are developing a TBV against Pvs25, the *P. vivax* homolog of Pfs25, in parallel with the Pfs25 TBV [5], and there is no evidence to suggest that one TBV will be effective against the other *Plasmodium* species.

Box 3

Human malaria parasite species

Plasmodium falciparum

Distributed across the globe, especially in Sub-Saharan Africa. Primary cause of malaria-associated mortality.

Plasmodium vivax

Focal distribution in Asia, Central and South America and the Western Pacific. Primary cause of malaria-associated morbidity and relapsing disease.

Plasmodium ovale

Occurs across much of Asia and the Western Pacific. Can cause relapsing malaria and is associated with widespread morbidity and some documented mortality.

Plasmodium malariae

Patchy, widespread distribution in Asia and the Western Pacific Islands and low prevalence in South America. Causes mild disease and recrudescent malaria for more than one year up to the lifetime of the host.

The rapid progression of TBV prototypes to clinical studies suggests that there is avid support for this approach. The groups that have led the development of these vaccines have started addressing many of the intrinsic obstacles associated with TBVs, including poor immunogenicity of the Pfs25 and Pvs25 antigens [6] and the potential for polymorphism among parasite populations in different geographical sites [7–9]. To address these issues, alternative prime-boost strategies have been adopted and have yielded significantly more immunogenic formulations [6,10–17]. The issue of polymorphism or clonal variability among different *P. falciparum* or *P. vivax* strains, however, remains a palpable problem. The earlier work had used laboratory strains of *P. falciparum* or *P. vivax* to test the efficacy of the TBVs in laboratory colonies of *Anopheles*. Analyses of Pfs25 polymorphism in field

isolates from Southeast Asia and Brazil have been conducted, and unlike Pvs25, Pfs25 was less polymorphic [8]. Moreover, field tests of the efficacy of both Pfs25 and Pvs25 TBVs against local parasite strains were promising [18,19]. However, the degree of Pfs25 polymorphism occurring in Sub-Saharan Africa remains unclear. The potential attenuating effect that could result from immune pressure exerted against the parasite in the mosquito can be determined only by a longitudinal study. Although the importance of this issue is controversial, it is especially salient given that one of the main hurdles to sexual- or sporogonic-stage TBVs is the inherent need for copious amounts of antibody to completely block parasite development in the mosquito [20]. This is a notable challenge in malaria-endemic regions, not just for TBVs but for any vaccine because of immunosuppression of hosts by HIV or unrelated pathogen comorbidity. The requirement for high-antibody titers in this context could exert a limit on the utility of the current parasite-based TBV formulations.

Vector-based TBVs

Given the relative success of targeting parasite surface molecules in TBVs, why would one target molecules on the mosquito vector as an alternative approach? The answer is that the arthropod midgut acts as both a common pathogen barrier and a universal pathogen gateway [21] and, as such, the mosquito-vector-based approach offers comparable benefits to and several advantages over the parasite-based TBV approach.

It is known that not all microbial pathogens can establish infections in the midgut of blood-feeding arthropods, and those that are successful in establishing an infection appear to use a conserved strategy for midgut invasion. To invade or establish in the arthropod, microbes target the most abundant ligands that are present on the luminal surface of the midgut, which faces the bloodmeal [21]. For example, *Borrelia burgdorferi* uses its outer surface protein (OspA) to bind to a ligand (tick receptor of OspA, or TROSPA) that is present in the midguts of ixodid ticks to adhere to the tissue [22], and *Leishmania* parasites attach to galectins on the sandfly midgut to prevent expulsion from the midgut during defecation [23].

In the context of malaria transmission, recent evidence suggests that *Plasmodium* parasites use multiple mosquito midgut molecules as adhesion ligands, which include glycans (carbohydrates) [24–28] and enzymes, such as alanyl aminopeptidase (APN) [29], that are involved in blood digestion. Additional glycoproteins mediate *Plasmodium* parasite invasion of the midgut, but the identities of these ligands are unknown [30,31]. Other molecules, such as carboxypeptidase B (CPB) [32], can act as ‘enabling factors’ and perhaps process crucial parasite molecules involved in cell invasion. In every case when antibodies raised against known or unidentified midgut ligands were included in an infective bloodmeal, the relevant vectors were ‘passively immunized’ (i.e. the development of pathogens was inhibited significantly by the antibodies) [22–26,29–32]. At least in the case of both murine and human *Plasmodium* antibodies, the inhibitory activities of these antibodies appear to be restricted to specific antigens because monoclonal antibodies against other antigenic mosquito midgut glycans or glycoproteins do not confer transmission-blocking immunity (R.R.D., unpublished). Therefore, mosquito-based TBVs can be just as effective as parasite-based TBVs in disrupting parasite transmission.

Parasite attachment ligands on the mosquito midgut as TBV target antigens

The antibody-mediated inhibition of the interaction between the sandfly midgut galectin and glycans on the *Leishmania* promastigote flagella is straightforward. Masking of this necessary protein–glycan interaction prevents the parasites from attaching to the midgut. By contrast, *Plasmodium* ookinete interaction with the mosquito midgut surface is complex, wherein multiple ligands are co-opted during the process of midgut invasion. To date, several ookinete surface or secreted molecules have been shown to be crucial for successful attachment to and invasion of the midgut: circumsporozoite and thrombospondin-related anonymous protein (CTRP), chitinase, von Willebrand Factor A-domain related protein (WARP), secreted ookinete adhesive protein (SOAP), two membrane attack ookinete proteins (MAOPs), cell-traversal protein for ookinetes and sporozoites (CelTOS), several lectin adhesive-like proteins (LAPs) and ookinete surface antigens (Pfs25 and Pfs28) (reviewed in Ref. [33]) [34–39]. Presumably, all these molecules are involved in the collective process of midgut invasion; however, only studies of CTRP, WARP and MAOP suggest a clear interaction between these molecules and the midgut apical surface [33–35]. Concrete identification of the cognate mosquito ligands that are not shared between humans and mosquitoes is lagging behind parasite surface molecule discovery. Only recently was a surface-expressed form of mosquito midgut calreticulin found to interact with Pvs25 *in vitro* [40], supporting the idea that Pfs25 and Pvs25 are involved in attachment [39]. It is unfortunate that it has not been determined whether antibodies against mosquito midgut surface calreticulin would confer transmission-blocking immunity and that the possible induction of autoimmune responses after mosquito calreticulin immunization has not been investigated, given that mosquito and human calreticulins share ~64% amino acid sequence identity [40].

The number of recently discovered ookinete ‘invasion molecules’ has been growing [20,21], bolstered in large part by proteomic studies [33]. Midgut cell invasion is a multistep process, and, thus, the discovery of numerous adhesion and invasion molecules is not too surprising. These molecules have not been assigned roles in the specific steps of the invasion process, which includes midgut cell recognition, attachment, gliding, apical orientation, membrane disruption, cell entry, cell traversal and egress on the basal side. Ideally, several sequential steps should be concomitantly targeted to achieve maximum inhibition and reduce the chance of parasites evading the imposed barrier. Preventing the first step of ookinete attachment to the midgut necessarily will preclude the secondary course of events, and, as such, is an attractive target. However, the sheer number of parasite molecules that presumably are involved in this initial process raises the possibility that targeting one mosquito molecule alone is unlikely to confer complete transmission-blocking immunity. Two recent reports provide evidence in support of this assertion. Antibodies against specific targets such as APN [29] or CPB [32] confer significant but incomplete transmission-blocking immunity against *P. falciparum*. There are at least three possible explanations for these independent observations: (i) CPB and/or APN are only indirectly involved in ookinete invasion of the midgut, (ii) APN is part of a larger molecular complex with other unknown mosquito ligands on the midgut epithelial surface and (iii) there is considerable

plasticity regarding the route of invasion and CPB and APN represent only two (of several) interaction pathways for the ookinete.

These issues are difficult to address, especially because it is not clear at what step of the ookinete invasion process these two molecules play a role and their interactions have not been mapped to a specific parasite molecule. It can be argued that APN does not interact directly with any parasite molecule and that antibody-mediated inhibition is a result of an indirect mechanism (e.g. blocking access to an adjacent molecule on the midgut that is the real ligand for the parasite). The discovery of APN was by virtue of its interaction with the lectin jacalin, which has been shown to be a potent inhibitor of ookinete attachment to midgut microvilli [27]. Because antibodies against APN further corroborate the inhibitory effect of jacalin [27], APN is presumably involved in ookinete attachment [29]. CPB was chosen as a target by virtue of its observed activity early in the blood-feeding process of the mosquito. Because anti-CPB antibodies appear to disrupt enzymatic activity, it was suggested that CPB is not an attachment ligand but, instead, processes parasite zymogens that are involved in midgut cell invasion [32]. Alternatively, CPB could mediate a mosquito cellular process that the parasite needs to invade the midgut epithelial cell.

The prospect that several midgut molecules are co-opted during the invasion process has not been investigated thoroughly, but the fact that multiple parasite molecules are required across overlapping steps during midgut invasion [33–35] gives some credence to this hypothesis. Pull-down experiments, which have not yet been performed, potentially could identify midgut molecules or parasite molecules that interact with APN or those parasite proteins that are cleaved by CPB. In general, anti-APN antibodies appear to be more effective in blocking parasite invasion than anti-CPB antibodies [29,32], which could suggest a hierarchical process in the use of mosquito molecules by ookinetes, wherein anti-CPB-antibody-mediated inhibition can be circumvented by the parasite. Anti-APN antibodies might then act at a later, more crucial, step wherein there is less room for the parasite to get around the blockade. This difference in inhibitory activity also might just reflect the quality of the antibody and/or the antigen that was used in generating the antibodies.

Taken together, these observations emphasize that multiple mosquito ligands will need to be targeted to confer complete transmission-blocking immunity. To date, there are only three studies that have shown clearly that multivalent targeting can achieve this goal. In one study the authors compared the transmission-blocking efficacy of monoclonal antibodies that target either a midgut peptide or a glycan epitope in membrane-feeding assays [31]. These authors reported inhibition of *P. falciparum* and *P. vivax* oocyst development between 85% (for anti-peptide antibodies) and 100% (for anti-glycan antibodies). A second study showed that another monoclonal antibody against a conserved midgut surface glycan epitope [25] completely blocked *Plasmodium yoelii* (a murine malaria parasite) development in mosquitoes [24], and indirect evidence indicates that it also would be effective against *P. falciparum* [25,29]. The third study used a commercial IgM antibody against chondroitin sulfate, which is present on midgut microvilli surface proteoglycans [26]. The blocking efficacy of the IgM antibody was incomplete (close to 80% inhibition); however, RNA interference-mediated knockdown of chondroitin sulfate biosynthesis in the mosquito

resulted in 95% inhibition of *P. falciparum* development. These data suggest that glycosaminoglycans are important for ookinete attachment to the midgut, an adhesion mechanism that appears to be conserved among several vector-borne pathogens [21].

Although monoclonal IgG1, IgG2a and IgG3 antibodies have been generated against midgut glycan epitopes previously [25,31], glycans generally are considered to be T-cell-independent antigens and, thus, poor immunogens incapable of eliciting immunological memory. However, recent advances in the design of glycan antigens have yielded tremendous success (reviewed in Ref. [41]). For example, semi-synthetic glycan-based vaccines against bacterial and viral pathogens [42–44] and tumor cells [45] have been developed, and the use of peptide mimotopes as surrogate immunogens for poorly immunogenic glycan antigens has been considered to drive a T-cell-dependent response [46,47].

Antibodies directed against glycan epitopes epitomize the multivalent TBV approach. The antibodies target highly conserved glycans on several glycoprotein ligands present on the midgut surface, which are purportedly used by ookinetes during midgut invasion. Consequently, it is plausible that one mosquito-based multivalent TBV will be highly effective because it can shut down multiple ‘gateways’ used by different malaria parasite species. More importantly, multivalent TBVs are likely to be more effective than TBVs relying on a single antigen and, in turn, would reduce the risk of selecting for resistant parasites.

The mosquito midgut antigen advantage

One of the biggest advantages of targeting mosquito mid-gut ligands is that it obviates the need to develop separate vaccines that target each of the parasite species. *P. falciparum* and *P. vivax* (along with the other two human malaria parasites, *Plasmodium ovale* and *Plasmodium malariae*) are transmitted exclusively by anophelines. From an evolutionary perspective *Plasmodium* parasites have developed a strategy to use a defined (i.e. conserved) set of ligands that are present across a specific subset of anopheline midguts. Therefore, by developing TBVs against these antigens, we theoretically can block all malaria parasite species across all anopheline vector species. Lal *et al.* has already shown that this is possible [30]. These authors showed that when monoclonal antibody MG2B was fed to mosquitoes in bloodmeals infected with *P. falciparum*, *P. vivax* or *P. yoelii*, it strongly inhibited parasite development in the mosquito. Unfortunately, the identity of the MG2B epitope remains unknown. Furthermore, anti-APN antibodies that are effective against *P. falciparum* in *Anopheles gambiae* and *Anopheles stephensi* also are highly effective in blocking field isolates of *P. vivax* in *Anopheles dirus* (R. R. D., unpublished).

High anti-Pfs25 and anti-Pvs25 antibody titers are necessary to block parasite development [16,20]. A comparison of the quality of the binding affinity of the antibody and the minimum effective concentration required for the Pfs25, Pvs25 and APN TBV approaches is needed to set standards for TBV efficacy. This is difficult because binding affinities of the antibodies have never been determined and the method for estimating the concentration of antibody used in membrane-feeding assays varies among investigators. Anti-AgAPN1

antibody concentrations appear to be effective between 10–500 $\mu\text{g ml}^{-1}$ [29]. Couple this benefit with the observation that specific midgut antigens are highly immunogenic alone and are easily expressed as recombinant proteins with other carriers, such as tetanus or cholera toxin, to increase immunogenicity further. One then can envision augmenting the efficacy of TBV formulations currently under development by combining parasite antigens such as Pfs25 and Pvs25 with mosquito midgut antigens in a single formulation, thereby minimizing the possibility of parasite escape.

Other potential vector-based TBVs

Mosquito-life-shortening TBVs

Targeting midgut antigens on mosquitoes that act as functional adhesion ligands for parasites is only one approach to the development of vector-based TBVs. Anti vector vaccines that not only block pathogen development but also limit the life span of mosquitoes below the crucial intrinsic incubation period of the pathogen and adversely affect fecundity also have been considered [31,32,48]. In the context of malaria, such a strategy would first block the parasite from invading the midgut; however, if parasite breakthrough occurs, then the mosquito also is prevented from living long enough (~14 days) to transmit the parasite to another host. Antibodies that also affect mosquito fecundity would decrease the subsequent vector population size. An additional feature would be that all mosquitoes that bite vaccinated individuals, regardless of their disease status, will suffer a similar fate. In essence this is an insecticide–vaccine. Although there have been antibody-mediated fecundity effects observed [31,32], antibodies capable of shortening mosquito life span have not been described to date.

Antisalivary-gland protein TBVs

A major disadvantage of TBVs is the lack of natural boosting. After vaccination, the host immune system would never again encounter mosquito-stage parasite surface antigens or vector midgut ligands. Therefore, another attractive approach to preventing pathogen transmission to mammalian hosts by arthropods involves targeting the vector's saliva proteins that are deposited into the skin at the bite site. This strategy would circumvent the 'lack of boosting' problem faced by the mosquito-midgut-based TBVs and prevent infection of the human host. The disruption of tick-borne pathogen transmission is a proof of concept, wherein antibodies raised against tick-saliva cement protein prevented mammalian host infection by *B. burgdorferi* [49]. In addition, mammalian hosts who are exposed to the bite of sandflies, or specific sandfly saliva antigens, are protected from leishmaniasis [50,51], and hosts pre-exposed to mosquito bites had significantly less malaria disease burden, as predicted by the 'pre-exposure' hypothesis [52]. Clearly, some amplification of the protectiveness of this approach is needed, given that natural exposure to mosquito bites is not protective in disease-endemic countries. Recent proteomic analysis confirmed five new proteins that are present specifically in saliva and identified another 30 putative saliva proteins. The proteins' role during sporozoite deposition in the dermis and their suitability as targets for anti-saliva protein TBVs remains to be determined [53].

Concluding remarks

The canonical approach to TBV design focuses on identifying immunogenic molecules that are present on the pathogen; however, this by no means represents the only viable approach to curbing the malaria problem. As a complementary line of attack, we can target molecules on the mosquito midgut and/or in the saliva that facilitate parasite cell invasion of the vector and mammalian host, respectively. The development of parasite-based TBVs is well in advance of the approach described in this review, yet mosquito-based TBVs will maintain substantial utility as a means to block parasite escape mutants, which could evolve as a result of the implementation of malaria vaccines against the liver-, red blood cell- or sexual-stage antigens (Box 2). Elucidating the role of the recently identified and characterized mosquito midgut and salivary molecules in parasite transmission biology is necessary to advance this approach further.

With the advance of the Pvs25 vaccine toward Phase II clinical trials and the recent success of the RTS,S vaccine in Mozambique, we have great hope that we will see a multistage malaria vaccine in the near future (Figure 1) that encompasses transmission-blocking antigens (both parasite and mosquito), thereby bolstering the highly efficacious use of insecticide-treated bed nets and ultra-low volume spraying of DDT.

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Glossary

Blood-stage vaccines	vaccines in this group are ‘curative’ because they aim to attenuate the severity of disease in infected individuals by preventing the cyclical invasion of red blood cells by merozoites
Exflagellation	male gametocytes extrude eight haploid microgametes (flagella), which fertilize the female macrogamete. These emerging microgametes sequester female macrogametes around themselves into so-called exflagellation centers
Liver-stage vaccines	vaccines in this group aim to prevent liver infection by sporozoites and/or inhibit development of the parasite in the liver. These vaccines are ‘preventative’ in that they prevent the vaccinated individual from contracting disease
Membrane-feeding assay	an experimental method for testing the efficacy of antibodies generated against both parasite and mosquito antigens in blocking transmission of the pathogen through the insect vector. To conduct the assay, infected blood is mixed with antibodies and placed in a special water-jacketed apparatus from which mosquitoes feed on the blood–antibody mixture through a thin membrane

Transmission-blocking vaccines

unlike the canonical vaccines described above, which protect the vaccinee, vaccines in this group do not protect the vaccinated individual from infection. These ‘alternative’ vaccines aim at preventing the spread of the pathogen from an infected individual to a noninfected individual, eliciting herd immunity if a large proportion of the target population is immunized

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Figure 1.

Malaria vaccines target different stages of the parasite life cycle. Liver- and blood-stage vaccines target parasites developing within the mammalian host, whereas transmission-blocking vaccines (TBVs) aim to block the parasite development in the mosquito. See Box 1 for a description of the parasite life cycle. Mosquito stages of the parasite are shown clockwise from right to left, beginning with Giemsa-stained male (pink) and female (blue) gametocytes, Giemsa-stained ookinetes, Mercurochrome-stained oocysts on the outside of the mosquito midgut, and immunofluorescence detection of circumsporozoite protein on the surface of a salivary gland sporozoite. The mosquito images were modified from the original produced by James Gathany for the CDC Public Health Image Library (<http://phil.cdc.gov/phil/home.asp>).