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Review article

Apical Membrane Antigen 1 (AMA1) as a Vaccine Target Against *Plasmodium knowlesi*: Opportunities and Challenges

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Abstract: *Plasmodium knowlesi,* the fifth human malaria parasite, has led to an emerging health crisis in Southeast Asia. *P. knowlesi* is an ideal vaccine target for its integral membrane protein, apical membrane antigen 1 (AMA1), which is critical for merozoite invasion of erythrocytes. Challenge studies in rhesus macaques show that recombinant PkAMA1 can elicit protective immunity and growth-inhibitory antibodies. The genetic diversity of PkAMA1 is lower than that of *P. falciparum* AMA1, which may facilitate the design of vaccines that are simpler and can induce cross-species protection. Some challenges include the need for high antibody levels, the possibility of strain-specific immune responses, and manufacturing complexity. This review covers the structural biology of PkAMA1, preclinical evidence supporting its potential as a vaccine target, and opportunities and strategies to make it a clinically viable intervention.

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Introduction

Malaria continues to be a major cause of deaths and morbidity from infectious diseases worldwide. *P. falciparum* and *P. vivax* are the most common malaria species causing infections in humans globally, but *P. knowlesi,* previously associated with a simian sort of zoonotic parasite, has recently arisen as the dominant malaria species in Malaysian Borneo and a major species in Southeast Asia [1]. *P. knowlesi* has a rapid 24-hour erythrocytic cycle and can cause a rapidly progressive, severe, and fatal disease in humans [2]. However, although *P. knowlesi* is clinically relevant, it is far less studied in global vaccine research.

The blood-stage antigens are promising targets for a malaria vaccine because immunity to the blood-stage parasite can delay parasitaemia and improve clinical disease management. The apical membrane antigen 1 (AMA1) has one of the longest research histories among blood-stage candidates. AMA1, a conserved region across Plasmodium species, plays a vital role in merozoite invasion and is highly immunogenic, eliciting antibodies with *in vitro* and *in vivo* growth-inhibitory activity [3, 4]. The review assesses PkAMA1 as a vaccine candidate, summarizing the available information about its biological function, preclinical efficacy, genetic properties, and major opportunities and challenges that outline its vaccine development trajectory.

Biology of AMA1

AMA1 belongs to a family of proteins that are type I integral membrane proteins and are expressed in the micronemes of Plasmodium merozoites [5, 6]. It has an ectodomain divided into three distinct subdomains (domains I, II, and III), each of which is bound by several disulfide bonds that are essential for maintaining the conformational epitopes of the invasion-inhibitory antibodies[7]. AMA1 is rapidly surface translocated from microneme stores upon merozoite egress from ruptured schizonts, undergoing proteolytic processing that yields an approximately 83-kDa precursor that produces a mature membrane-associated form of about 66 kDa, which participates in the formation of

the AMA1–RON moving junction required for erythrocyte invasion [4, 8].

The mechanistic insight reveals that the tight moving junctions and the formation of ring-like structures facilitating parasite invasion of host cells are mediated by AMA1 via erythrocyte surface apposition of merozoites, as illustrated by Figure 1. This is achieved through physical contact with the secreted erythrocyte membrane protein rhoptry neck protein 2 (RON2), which is a receptor for AMA1[7, 9]. Further regulation is carried out by Protein kinase A-dependent phosphorylation of AMA1[10]. Failure to generate viable AMA1 knock-out parasites in several different species of Plasmodium supports the evidence that AMA1 is necessary for blood-stage survival[4, 11]

Domain I of AMA1 bears the greatest sequence variability across parasite populations and contains key targets of invasion-inhibitory antibodies that map to a structurally defined binding hot spot[12, 13]. These antibodies block the AMA1-RON2 interaction or sterically obstruct invasion, thereby making them directly relevant to vaccine-induced protection[14, 15]. Domains II and III are comparatively more conserved and may harbour cross-species epitopes relevant to multi-species vaccine design [16]. Importantly, AMA1 is expressed at relatively high density on the merozoite surface, ensuring adequate exposure to vaccine-induced antibodies during the brief extracellular merozoite phase, a kinetically constrained window of immune vulnerability that effective blood-stage vaccines must exploit[7].

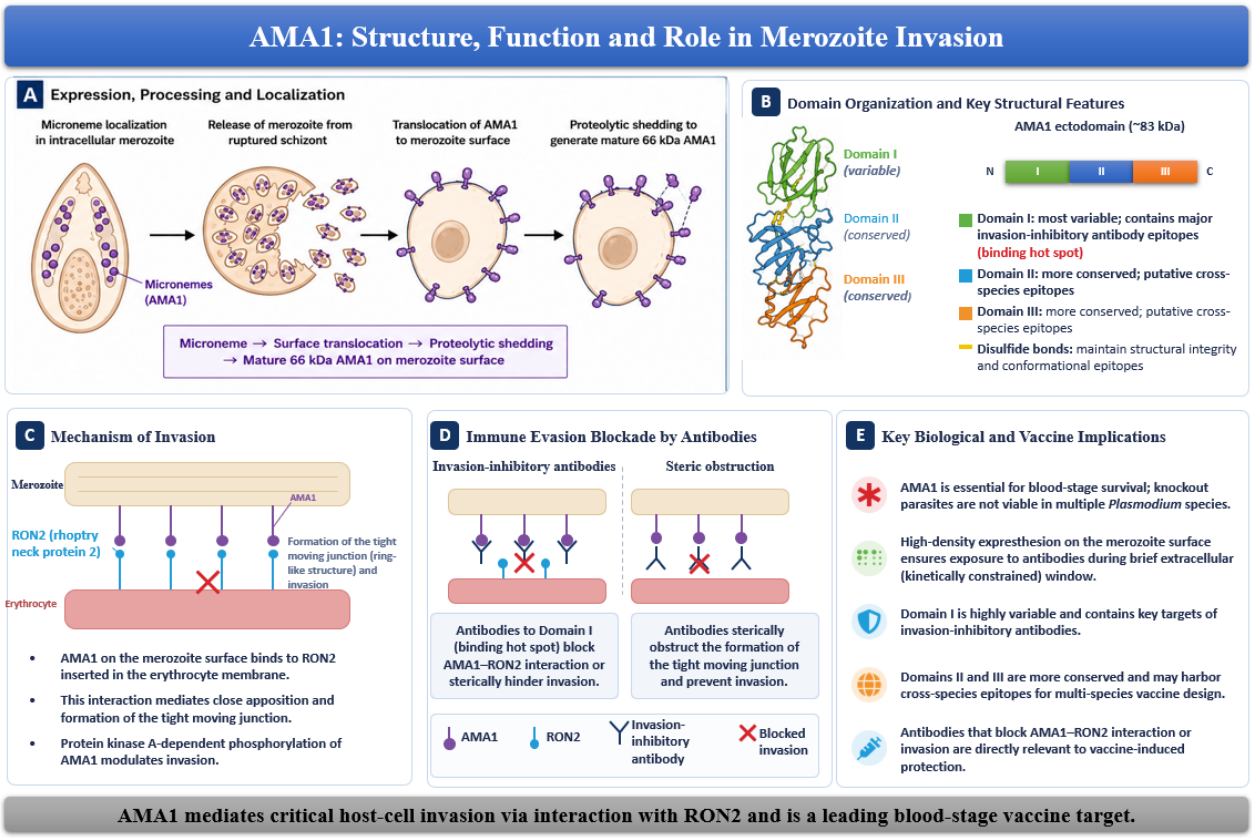

Figure 1. Structure, function, and vaccine relevance of AMA1. AMA1 moves from micronemes to the merozoite surface, where its interaction with RON2 supports tight-junction formation and erythrocyte invasion. Antibodies can block this interaction or sterically prevent invasion, making AMA1 an important blood-stage malaria vaccine target.

PkAMA1: Structure, Diversity, and In Vitro Activity

The 3D crystal structure of *P. knowlesi* AMA1 domains I and II gave a detailed and comprehensive insight into the domain organization, surface-exposed regions, and conserved hydrophobic cleft of these domains. Structural analysis of PkAMA1 bound to the invasion-inhibitory monoclonal antibody R31C2 also revealed that a loop in domain II of PkAMA1, along with neighbouring loops, forms a conformational epitope that can be accessed by the antibody, which gives insight into the mechanism of antibody-mediated inhibition of erythrocyte invasion[17]. Structural characterization of PkAMA1 in complex with an

invasion-inhibitory monoclonal antibody revealed that functionally relevant epitopes on the surface of PkAMA1 are accessible to the humoral immune system and elucidated the structures of PkAMA1 and its interactions with the antibody, thereby explaining its invasion-inhibitory activity [17, 18]. The structure is of critical importance for structure-based rational vaccine design.

The major characteristic of PkAMA1 compared with *P. falciparum* is its low genetic diversity. The gene PkAMA1 is known to have low polymorphism throughout Peninsular Malaysia and Sabah and is not subject to diversifying (positive) selection [19-22]. This diverges significantly from AMA1 of *P. falciparum*, which demonstrates comprehensive polymorphism in domain I due to continued immune selection in endemic populations [23]. PkAMA1 full-length sequencing from clinical isolates revealed a limited number of circulating haplotypes [21]. The limited variation is due to the recent emergence and transmission of *P. knowlesi* in humans, thereby simplifying antigen selection for a vaccine.

The functional relevance of PkAMA1-directed antibodies has been confirmed by in vitro studies using a human erythrocyte-adapted strain of *P. knowlesi* (PkAMA1-H.1). This system is highly sensitive to anti-PkAMA1 antibodies, which potently block invasion of human red blood cells. Of interest, in a synergistic fashion, anti-PkAMA1 combined with anti-*P. knowlesi* Duffy binding protein alpha (PkDPBα) gave significantly greater inhibitory effect than either antibody alone [24], emphasizing the importance of multiple antigen vaccine strategies. Furthermore, bioinformatics analyses showed that conserved sequence regions in PkAMA1 peptides that contain B-cell and T-cell epitopes are also suitable for multi-epitope immunogen design[25, 26].

Opportunities as a Vaccine Target

Several experimental and literature-based studies confer PkAMA1 as a promising vaccine candidate. The most direct evidence was obtained from rhesus macaque challenge studies where animals immunized with recombinant PkAMA1 formulated with the adjuvant CoVaccine HT exhibited growth-inhibitory antibodies that were linked to control of blood-stage parasitemia[27, 28]. One of 6 inoculated animals completely controlled parasitemia upon challenge, whereas four of 6 controlled infections after boosting and re-challenge. Protection was associated with levels of growth inhibition assay (GIA) IC50 (the inverse of *in vivo* parasite growth rates), which gives GIA the potential to serve as a useful surrogate efficacy marker for future testing [27]. The primate model data are essential due to the physiological and immunological relevance of the primate model to the human disease context, as rhesus macaques are the natural reservoir host for *P. knowlesi*. Table 1 summarizes AMA1 biological significance and vaccine potential against *P. knowlesi.*

More extensive comparative evidence supports AMA1as a potential vaccine candidate. Recombinant *P. falciparum* AMA1 has protected Aotus vociferans monkeys against blood-stage challenge [29], and AMA1 vaccination has conferred sterile immunity to sporozoite challenge in rodent studies [30]. *In vitro* growth inhibition assays have consistently shown that antibodies against AMA1 are more functional than other blood-stage antibodies, such as anti-MSP1, anti-LSA1, and anti-RBP2 [31].

One of the most important characteristics of PkAMA1 is its low polymorphism. The allelic diversity has repeatedly hampered clinical AMA1 vaccine trials in *P. falciparum* by inducing strain-specific immune responses that lack cross-coverage [20, 32, 33]. A single-allele or small number of alleles vaccine would be sufficient to protect against most circulating strains due to the limited haplotype diversity of AMA1, which is a huge benefit in vaccine design and manufacturing processes.

The conservation of AMA1 epitopes across various Plasmodium species offers another opportunity. Anti-AMA1 antibodies have been found to cross-react with AMA1 from other species in cross-reactivity studies [16, 34, 35]. Multiple species protection with a single immunogen, as *P. knowlesi* co-circulates with *P. vivax* in some transmission settings and *P. falciparum* throughout Southeast Asia, is a public health advantage that vaccines based on conserved cross-species epitopes in these parasites may provide due to cross-reactivity.

Challenges and Limitations

Despite its promising profile, PkAMA1-based vaccine development faces substantive challenges. AMA1-targeting vaccines are titer-dependent: they need high antibody levels to reduce parasite growth *in vivo*, as shown in studies in Aotus primates [36]. Such titres will require optimization of the adjuvants, multiple doses, and perhaps new delivery systems in different human populations.

A concern about strain-specific immune responses remains. This has been demonstrated using *P. falciparum* antibodies raised against AMA1 that poorly inhibit AMA1 from a parasite with a different allele than homologous antibodies, with heterologous

inhibition always less than homologous inhibition [37, 38]. This likelihood is significantly lower in PkAMA1 but might still be reduced owing to residual allelic variation if vaccine immune responses are strain specific. For broad protection, one or more of the following strategies must be adopted: immunodampening strategies [39, 40] and structure-based rational antigen engineering [35].

Table 1. Biological significance, vaccine potential, and challenges of AMA1 as a vaccine target against *Plasmodium knowlesi.*

AMA1 Property	Biological Significance	Evidence in *P. knowlesi*	Vaccine Opportunity	Major Challenge	References
Essential role in erythrocyte invasion	AMA1 interacts with RON2 to form the moving junction required for merozoite invasion	Conserved RON2-binding interface in PkAMA1	Blocking AMA1–RON2 inhibits invasion	Alternative invasion pathways may compensate	[17, 41]
High immunogenicity	Induces strong humoral and cellular immunity	Recombinant PkAMA1 elicits invasion-inhibitory antibodies	Suitable for recombinant and nanoparticle vaccines	Immunity may decline without boosters	[27, 42, 43]
Conserved three-domain structure	Three disulfide-rich domains preserve epitopes	Crystal structures show conserved architecture	Supports structure-guided design	Variable loops promote immune escape	[17, 44]
Genetic polymorphism	Most polymorphisms occur in Domain I	Binding pocket relatively conserved	Multi-allele vaccines may broaden protection	Allele-specific immunity	[5, 42, 45]
Compatibility with modern vaccine platforms	Expressed as recombinant protein, DNA, viral vector and VLP	PkAMA1 formulations induce strong responses	VLPs enhance immune memory	Optimal formulation unknown	[43, 46]
Cross-species vaccine relevance	Conserved across Plasmodium spp.	Similarity with *P. falciparum* and *P. vivax* AMA1	Supports pan-malaria vaccines	Cross-protection incomplete	[4, 43]
Evidence from preclinical studies	Vaccination reduces parasitemia	Protection shown in rhesus macaques	Proof-of-concept	No human trials	[17, 27]
Clinical translation potential	Knowledge from *P. falciparum* informs PkAMA1	Increasing zoonotic cases justify development	Existing technologies accelerate progress	No efficacy trials; antigenic diversity	[43, 47, 48]

The manufacturing sector is an additional challenge. Protein folding and the formation of disulfide bonds in recombinant expression systems are important to maintain conformational epitopes that generate invasion-inhibitory antibodies [49, 50]. The quality and function of antibody responses are affected by post-translational modifications, such as glycosylation, which vary between expression systems [51]. Therefore, it is a non-trivial manufacturing challenge to ensure consistency between research-grade and clinical-grade material.

Another constraint is the lack of knowledge of the full extent of heterologous immunity against PkAMA1 in humans. The association between naturally acquired antibody and protection has not been formally established, although IgG responses to PkAMA1 can be detected in 46.7% of naturally infected patients [24]. Malaria caused by *P. falciparum* has been studied by natural exposure for decades, resulting in the definition of seroepidemiological correlates which provide benchmarks for the vaccine. By contrast, the natural exposure dynamics of *P. knowlesi* infection have complicated the interpretation of naturally acquired immune responses. Currently, there are no human correlates of protection in *P. knowlesi* vaccine development, and further research into these relationships is a priority in the field.

Future Directions

There are several promising strategies for the further development of PkAMA1 vaccines. The crystal

structure of PkAMA1 and the structure, interactions, and conservation of the AMA1-RON2 interface can guide the design of new immunogens that direct immune responses to the most functionally essential and conserved epitopes [12, 17]. Bioinformatics-based identification of B-cell and T-cell epitopes is a lopsided route to breadth and cross-strain protection with multi-epitope vaccines [25]. Identification of strain-specific or weakly inhibitory antibody responses to further immunodominant polymorphic AMA1 epitopes can help to design immune-focused vaccine constructs. The inclusion of these variable regions in a vaccine could shift antibody responses towards conserved, functionally important epitopes, thereby enhancing the vaccine's breadth of inhibition. This approach has yet to be tested for PkAMA1, however. [14].

The *in vitro* synergy observed with the multi-antigen vaccines, especially with co-immunization of PkAMA1 with PkDBPα, is highly encouraging and should be given a high priority on the preclinical testing agenda [24]. In addition, novel platforms like viral-vectored vaccines, virus-like particles, and mRNA formulations have demonstrated immunogenicity benefits over recombinant proteins, with preliminary *in vivo* data demonstrating the promise of cytomegalovirus (CMV) vectors expressing *P. knowlesi* antigens [52][36] and mRNA vaccines showing improved immunogenicity of AMA1 in other Plasmodium species.

Conserved cross-species AMA1 epitopes could target *P. knowlesi, P. vivax* and P. *falciparum* at the same time [16, 35]. Studies of controlled human *P. knowlesi* malaria infection (CHMI) is an efficient avenue for identification of immunological correlates of protection and screening formulations before field trials. Taken together, these strategies provide an abundance of translation opportunities to bring the biological promise of PkAMA1 to clinical impact.

Conclusion

AMA1 is a validated, structurally defined, and immunologically tractable target to develop a vaccine against P. knowlesi malaria. Overall, these features make PkAMA1 one of the most promising blood stage vaccine candidates for this emerging pathogen. Antibody requirements, strain specificity, and manufacturing complexity are challenges that exist and can be overcome with rational design and innovation of platforms. *P. knowlesi* is expanding its role as an important human pathogen in Southeast Asia, and continued investment in PkAMA1 vaccine research provides a credible and scientifically sound avenue to an effective preventative intervention.

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