Functional conservation of the AMA1 host-cell invasion ligand between

*P. falciparum* and *P. vivax*: a novel platform to accelerate vaccine and drug
development

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**Running Title:** Functional conservation of AMA1

**Brief Summary:** Malaria vaccine candidate AMA1 of *P. vivax* can functionally complement
*P. falciparum* AMA1 in erythrocyte invasion, demonstrating remarkable molecular
flexibility. Modified *P. falciparum* expressing PvAMA1 provides a key new platform for
quantifying the invasion-inhibitory capacity of antibodies and small molecules to accelerate
development of vaccines and therapeutics for *P. vivax* malaria.
ABSTRACT

P. vivax and P. falciparum malaria species have diverged significantly in receptor-ligand interactions and host-cell invasion. One protein common to both is the merozoite invasion ligand AMA1. While the general structure of AMA1 is similar between species, their sequences are divergent. Surprisingly, it was possible to genetically replace PfAMA1 with PvAMA1 in P. falciparum parasites. PvAMA1 could complement PfAMA1 function and supported efficient invasion of erythrocytes by P. falciparum. Genetically-modified P. falciparum expressing PvAMA1 evaded the invasion inhibitory effects of antibodies to PfAMA1, demonstrating species-specificity of functional antibodies. We generated antibodies to recombinant PvAMA1 that effectively inhibited invasion, confirming the function of PvAMA1 in genetically-modified parasites. Results indicate significant molecular flexibility in AMA1 enabling conserved function despite substantial sequence divergence across species. This provides powerful new tools to quantify the inhibitory activities of antibodies or drugs targeting PvAMA1, opening new opportunities for vaccine and therapeutic development against P. vivax.

Key words: Plasmodium falciparum, Plasmodium vivax, malaria, AMA1, Ron2, Merozoite invasion, vaccine.
BACKGROUND

There is a renewed focus on *P. vivax* as a result of a growing understanding of the burden of disease, including its role as a major cause of severe malaria and chronic illness [1] [2]. Around 2.5 billion people are at risk of *P. vivax* malaria globally [3]. Because of its dormant hypnozoite liver stage, *P. vivax* can cause repeated relapses over time, even in the absence of exposure to infectious mosquitoes, as parasites are released from hepatocytes and replicate in the bloodstream leading to clinical illness and serious complications [4] [1] [2]. Therefore the development of vaccines that protect against the blood-stages of *P. vivax* is a priority. However, progress on *P. vivax* vaccine has been alarmingly slow, with extremely few candidates in development or in clinical trials [5].

*P. falciparum* and *P. vivax* are the principal species responsible for human malaria, but they are thought to have independently evolved from primate to human hosts [6] [7], which has led to them adopting different erythrocyte invasion mechanisms, including different invasion ligands and receptors [5]. Knowledge of specific molecular events that are shared or divergent between the two species is very limited. A fundamental roadblock in revealing molecular details is the inability to readily culture *P. vivax*. This has been possible for *P. falciparum* since the 1970s, but has proven a very major challenge for *P. vivax*. The lack of a culture system for *P. vivax* and capacity for genetic manipulation continues to pose a huge barrier to discovering *P. vivax* invasion events and biology and advancing vaccine development.

One invasion protein that is shared between *P. vivax* and *P. falciparum* is apical membrane antigen 1 (AMA1), which plays an essential role in infection of erythrocytes by the merozoite of *P. falciparum* [8] [9] and it is a leading vaccine candidate [10] [11] [12] [13] [14] [15]. In fact, AMA1 is common to all mammalian and avian malaria species, even though many other ligands and surface proteins are different between species. However, while the general three-domain structure of AMA1 is conserved among species, the
sequence varies considerably, including in key functional residues [16] [17]. A remarkable feature of AMA1 is that it utilises its own parasite-derived receptor complex for binding, known as the Ron-complex (composed of Ron2, 4, 5 and 8), rather than relying on pre-existing host receptors on the red cell surface. The Ron2 protein contains several internal transmembrane domains and upon translocation, inserts itself into the erythrocyte membrane, presenting a surface-exposed loop which interacts with the hydrophobic groove of AMA1 [18] [19]. This molecular event has been shown for *P. falciparum*, but not for *P. vivax*. The binding of AMA1 to its erythrocyte surface receptor, Ron2, is thought to initiate formation of the tight junction in the process of invasion [20] [21] [22] [23]. This provides a strong rationale for research to understand PvAMA1 function and how it has diverged from PfAMA1.

In this study, we aimed to understand the function of PvAMA1 and define functional divergence and conservation between *P. vivax* and *P. falciparum* AMA1 using molecular genetic approaches. We generated novel chimeric *P. falciparum* lines in which PfAMA1 was successfully genetically and functionally replaced with PvAMA1. This finding pointed to substantial molecular flexibility in the receptor-binding interactions and unexpected conservation of function between species. Our approach presents a solution to the major bottleneck of the inability to readily culture *P. vivax* to greatly facilitate vaccine and therapeutic development.
MATERIALS AND METHODS

Parasite culture, genotyping, and phylogenetic analysis

The origin of all wild type parasite lines used in this study has previously been described [24]. W2Mef parasite strains expressing W2Mef (W2-W2), 3D7 (W2-3D7) and FVO (W2-FVO) AMA1 alleles were generated in previous studies [24]. *P.falciparum* and *P.vivax* AMA1 protein sequences were obtained using NCBI BLAST and aligned using Clustal-Omega.

Generation of transgenic parasite lines

The hybrid allele of Palo Alto PvAMA1 (PvAMA1-T) was synthesised (Genescript) and codon-optimised for *E.coli*. It encodes the signal sequence and pro-domain of *P.falciparum* 3D7 AMA1 (aa 1-93) and the ectodomain (Ecto), transmembrane domain (TM) and cytoplasmic tail (CT) of the Palo Alto allele of PvAMA1 (Figure 1C). PvAMA1-T was transfected into W2Mef parental parasites as described [24]. Clonal populations were confirmed by Southern blot [24] (Figure 2A).

Invasion and growth assays

Invasion inhibition assays, with quantification by flow cytometry were performed as described [24] [25] [26]. Multiplication rates of transgenic parasites were quantified using flow cytometry over a single invasion cycle (N=6 wells per line). (Further details in Supplementary Methods).
Live-Cell Microscopy

Highly synchronous parasites were settled onto a 35 mm Fluorodish (World Precision Instruments) to produce a monolayer. Experiments were performed at 37°C with a Zeiss AxioObserver Z1 fluorescence microscope equipped with humidified gas chamber (94% N₂, 1% O₂, and 5% CO₂). Time-lapse videos were recorded with a high-resolution AxioCam MRm camera. ImageJ and Prism (Graphpad) were used to perform image and statistical analyses. Comparisons between groups was performed using t-tests (normally-distributed data) or Mann-Whitney U-test. For comparison of deformation scores between groups Chi-square tests were performed.

Expression of recombinant AMA1

Recombinant W2Mef, 3D7 and FVO AMA1 ectodomain protein (amino acids 25-546) were expressed in BL21 E.coli, purified and refolded as described [24] [27]. The DNA sequence encoding amino acids 25-546 of Palo Alto PvAMA1 was synthesised (Genescript) and ligated into the 6xHis expression vector pProEX HTb (Invitrogen). PvAMA1 expression, purification and refolding was performed as described for PfAMA1 [27].

Immunisation of rabbits and generation of antibodies

New Zealand White rabbits were immunised with 50 μg of recombinant PvAMA1 in Freunds adjuvant. Ethics clearance for rabbit immunisations was obtained from the Walter and Eliza Hall Institute Animal Ethics Committee, Australia. Antibodies to PfAMA1 were generated as previously described [24]. Anti-3D7 AMA1 and Anti-FVO AMA1 rabbit antibodies were tested as purified polyclonal rabbit IgG after purification using protein-G.
Due to limited supply, anti-W2Mef AMA1 rabbit IgG was not purified using protein-G and was tested as polyclonal anti-sera after heat inactivation for 45 minutes (56 degrees).

**Gel electrophoresis and western blots.**

SDS-PAGE and western blot analysis was performed as previously described [24]. Very late-stage schizonts containing merozoites were prepared by the addition of 1μM Compound-1 to 40h synchronised schizonts for 8 hours, as previously described [28]. Blots were probed with the relevant anti-AMA1 rabbit anti-sera or purified IgG. Blots were also probed with a mouse monoclonal antibody generated against the *P. falciparum* HSP-70 protein or rabbit anti-PfRON4.2 antibodies as a loading control.

**Indirect Immunofluorescence Assays (IFA)**

Thin smears of schizonts were air dried on microscopes slides, fixed in ice cold 10% Methanol / 90% Acetone for 5 minutes, air dried and then rehydrated in PBS for 10 minutes at room temperature (RT) before labelling with antibodies to PfAMA1-W2mef (1/250) or PvAMA1 (see Supplementary Methods).

**ELISA**

AMA1 proteins were coated at 1 μg/ml in PBS onto Maxisorp plates (Nunc) and antibodies were tested as described [29] (see Supplementary Methods).
RESULTS

Generation of transgenic *P. falciparum* lines that express *P. vivax* AMA1

Phylogenetic analysis of AMA1 genes from different *Plasmodium* spp. highlighted significant divergence between PvAMA1 and PfAMA1. PvAMA1 clustered with AMA1 species from simian *Plasmodium* spp., such as *P. knowlesi*, *P. coatneyi*, and *P. cynomolgi* (Fig 1A); whereas PfAMA1 clustered separately with the chimpanzee malaria, *P. reichenowi*. Amino acid sequence identity between PfAMA1 and PvAMA1 was 51% (Fig S1) [19]. Based on these findings, it was unclear whether PvAMA1 could complement the function of PfAMA1 in *P. falciparum*.

To attempt PfAMA1 replacement with PvAMA1, we used our previously described approach for generation of transgenic W2Mef *P. falciparum* lines that have the wild type PfAMA1 allele replaced with a codon-optimised PfAMA1 allele from other *P. falciparum* strains [24] [29]. The single cross-over approach results in the truncation of the WT PfAMA1 and the insertion a new PfAMA1 5’ promoter, codon optimised PfAMA1 allele and 3’ UTR immediately 3’ to the WT locus. This generates *P. falciparum* that expresses the newly inserted PfAMA1 allele and no longer expresses endogenous PfAMA1. Importantly, as the introduced PfAMA1 is controlled by a WT PfAMA1 promoter sequence, transgenic *P. falciparum* express equivalent amounts of the new PfAMA1 allele at the same developmental stages to that seen in WT *P. falciparum* and grow normally in extended *in-vitro* culture [24] [29].

We first attempted to replace the W2Mef AMA1 allele with a WT *P. vivax* AMA1 sequence (Palo Alto allele), but were unsuccessful (data not shown). A notable difference from PvAMA1 is that PfAMA1 has a pro-domain separating the signal peptide from Domain 1 of the PfAMA1 ectodomain; this is absent from PvAMA1 (Figure 1B). While the exact function of the pro-domain is not known, it is proteolytically cleaved from the 83 kDa
full-length PfAMA1 to yield a 66 kDa mature form of PfAMA1 [30]. The 83 kDa and 66 kDa forms of PfAMA1 both co-localise to the micronemes of late stage schizonts, but only the 66 kDa form appears to migrate to the merozoite surface to play a direct role in erythrocyte invasion [31]. While the role of the pro-domain is unknown, we hypothesised that the inclusion of the PfAMA1 pro-domain may be important for achieving expression of PvAMA1 in *P. falciparum*. Therefore, we constructed PvAMA1 allelic exchange vectors containing a hybrid Pf-PvAMA1 allele that contained the ATG start codon, Kozak sequence, signal peptide and pro-domain of PfAMA1 (3D7 allele), fused in-frame with the ectodomain, transmembrane domain and cytoplasmic tail of PvAMA1 (PvAMA1-T; Pulo Alto allele) (Figure 1B). This plasmid was transfected into the *P. falciparum* isolate W2Mef and successfully integrated into the WT AMA1 locus following drug selection (Figure S2A).

The loss of the endogenous PfAMA1 locus was confirmed by southern blot (Figure S2B) and expression of PvAMA1-T in the clonal *P. falciparum* line (named W2-PvAMA1) was confirmed by western blots probed with AMA1 antibodies (rabbit polyclonal anti-sera raised against recombinant PfAMA1:W2Mef allele), which cross-reacts with both W2Mef-PfAMA1 and PvAMA1 (Figure 2A). Bands corresponding to full length 83kDa and 66kDa forms of W2Mef-AMA1 were detected in non-reduced schizont extracts from parental W2Mef *P. falciparum*, but were not present in extracts from the W2-PvAMA1 *P. falciparum* clone, confirming the loss of endogenous W2Mef AMA1 expression. Instead, a single band corresponding to the 81kDa full length PvAMA1-T was detected in schizont extracts from W2-PvAMA1 *P. falciparum* (Figure 2A), suggesting that PvAMA1 had functionally replaced PfAMA1. The observation that full length PvAMA1-T (PvAMA1+Pf Pro-domain) migrated as a smaller (~2kDa) protein is consistent with its predicted molecular weight compared to full-length W2Mef AMA1. Polyclonal PvAMA1 IgG (R1387 &1388) partially cross-reacted with the native 83 and 66 kDa forms of W2Mef-PfAMA1 and the 81 kDa native form of PvAMA1 (Figure 2A). However, no other PvAMA1 bands were detected migrating less than the 81 kDa band in non-reduced schizont extracts from W2-PvAMA1 *P. falciparum*.
*P. falciparum* when probed with these antibodies (Figure 2A).

To determine if a smaller processed form of PvAMA1 equivalent to the pro-domain cleaved 66 kDa species of *P. falciparum* AMA1 [32], was produced in W2-PvAMA1 parasites, we treated parasite cultures with Compound-1 [28] (a trisubstituted pyrrole specific PKG inhibitor) to prevent schizont rupture and prepared extracts enriched with late-stage schizonts containing merozoites. When probed with polyclonal PvAMA1 IgG (R1387 &1388), 81 and 64 kDa species of PvAMA were detected in these schizont extracts, confirming that the pro-domain of the native PvAMA1 is cleaved prior to RBC invasion by W2-PvAMA1 *P. falciparum* (Figure 2B). Anti-3D7 AMA1 IgG cross reacted with the 81 kDa PvAMA1 species, but did not detect the smaller 64 kDa form.

**P. falciparum expressing PvAMA1 was not inhibited by antibodies to PfAMA1**

Imaging by immunofluorescence microscopy of fixed *P. falciparum* merozoites probed with either polyclonal anti-PvAMA1 or anti-W2Mef AMA1 antibodies confirmed that PvAMA1 was correctly localised to the apical end of merozoites in *P. falciparum* (Figure 3). This pattern of staining was also seen in IFA imaging of the control line W2-W2 *P. falciparum* (expresses PfAMA1) probed with either antibody and reiterates the cross-reactive nature of PvAMA1 and PfAMA1 antibodies, as was seen with western blots.

Importantly, the W2-PvAMA1 *P. falciparum* line completely escaped the inhibitory activity of anti-PfAMA1 antibodies tested in growth inhibition assays (GIA) (Figure 5B), which evaluated the ability of antibodies to inhibit invasion. In contrast, the same antibodies had a high level of invasion inhibition against parasites expressing W2Mef-PfAMA1. This further confirmed the loss of W2Mef-PfAMA1 expression and function, and the replacement with functional PvAMA1. Furthermore, results indicated that W2Mef AMA1 antibodies do not cross react with functionally important PvAMA1 epitopes, even though there is antibody
cross reactivity observed by western blotting (Figure 2A) and IFA of fixed merozoites (Figure 3).

Replacement of PfAMA1 with PvAMA1 did not affect *P. falciparum* multiplication rate or invasion kinetics.

Replacement of the endogenous PfAMA1 with PvAMA1 appeared to have no substantial impact on erythrocyte invasion and replication *in vitro*. No significant difference in the multiplication rates of *P. falciparum* engineered to express the W2Mef allele of PfAMA1 (W2-W2) or the Palo Alto allele of PvAMA1 (W2-PvAMA1) was detected during normal *in-vitro* culture, with both lines expanding approximately 8-fold each cycle (Figure S3). Using live cell imaging of invasion, where *P. falciparum* are cultured in a monolayer of erythrocytes, median invasion rates for W2-PvAMA1 *P. falciparum* were not significantly different to that of the control W2-W2-PfAMA1 line. (Figure S4). The PfAMA1-PfRon2 interaction has previously been reported to mediate formation of a tight junction between *P. falciparum* and the erythrocyte immediately prior to merozoite internalization [20].

Quantification of invasion kinetics and tight junction formation by live cell imaging did not detect any significant difference between the *P. falciparum* lines expressing PfAMA1 or PvAMA1 (Figure 4). Hence, PvAMA1 is able to mediate merozoite invasion efficiently, and similar to endogenous PfAMA1 when tested under *in-vitro* culture conditions, suggesting that PvAMA1 can effectively complement the function of PfAMA1.

Functional activity of antibodies generated against recombinant PvAMA1 protein

Antibodies were generated in rabbits immunised with refolded recombinant PvAMA1 ectodomain protein (rabbits: R1388 and R1387). Recombinant PvAMA1 (Palo Alto allele) was expressed in *E. coli*, purified, and then refolded by adapting protocols established for refolding of PfAMA1 (Figure S5). In invasion-inhibition assays, antibodies
raised to PvAMA1 specifically inhibited invasion of *P. falciparum* expressing PvAMA1, but had no inhibitory effect on invasion of *P. falciparum* expressing PfAMA1 alleles (Figure 5A). In fact, relative to control IgG, anti-PvAMA1 IgG appeared to slightly enhance the growth of *P. falciparum* lines expressing PfAMA1 alleles, and this enhancement was consistent across anti-PvAMA1 generated in two different rabbits. Confirming specificity, anti-W2Mef-AMA1 antibodies failed to inhibit invasion of *P. falciparum* expressing PvAMA1 to any extent, and also showed little cross-inhibition against *P. falciparum* expressing antigenically divergent *P. falciparum* AMA1 alleles, 3D7 or FVO (Figure 5B). Antibodies against the FVO-PfAMA1 allele showed minor cross-inhibition of *P. falciparum* expressing PvAMA1 (Figure 5D).

While antibodies to each of the PfAMA1 alleles generated a unique profile of inhibition across isolates, they all consistently inhibited invasion of the *P. falciparum* line expressing the homologous AMA1 allele the most (Figure 5B-D). In order to compare W2-PvAMA1 invasion inhibition by different antibodies, W2-PvAMA1 inhibition was normalised relative to the inhibition generated by each allele-specific antibody against the corresponding transgenic *P. falciparum* expressing the homologous PfAMA1 allele. When this was done, mean inhibition of W2-PvAMA1 parasites by W2Mef, 3D7 or FVO IgG was -6%, 7% and 36% respectively. These findings indicate that PvAMA1 functions to mediate invasion, and there is limited cross-inhibitory activity between antibodies to PfAMA1 and PvAMA1.

**Cross-reactivity of antibodies by ELISA and western blot**

Although AMA1 antibodies showed limited cross-species inhibition of invasion function, there was significant cross-reactivity to recombinant and native AMA1 proteins by western blot and ELISA. Antibodies raised to each of the three PfAMA1 alleles labelled reduced and non-reduced forms of native PvAMA1 and recombinant PvAMA1 protein by
western blot (Figure 6A). In ELISA, PfAMA1 antibodies cross-reacted with recombinant PvAMA1 protein, but at lower levels than PvAMA1 antibodies (Figure 6B).

Anti-PvAMA1 IgG also labelled recombinant PvAMA1 protein by western blot (Figures 6A). When measured by ELISA, PvAMA1 antibodies cross-reacted with all three recombinant *P. falciparum* AMA1 alleles tested (Figure 6C, D), being strongest for W2Mef-PfAMA1. This highlights differences in findings on antibody specificity when using standard immunoassays compared to functional inhibition assays, and has relevance to vaccine evaluation.

**DISCUSSION**

There is currently a very limited understanding of the roles of specific proteins in *P. vivax* merozoite invasion and functional conservation or divergence between *P. vivax* and *P. falciparum*. Using novel genetic approaches, we demonstrated that PvAMA1 can functionally complement PfAMA1 in *P. falciparum*. We demonstrated the successful replacement of endogenous PfAMA1 with a functionally complementary PvAMA1 via several means. Insertion of PvAMA1 and loss of PfAMA1 was confirmed by southern and western blots using probes or antibodies to AMA1, respectively. The loss of endogenous PfAMA1 was clearly demonstrated by the lack of invasion-inhibitory activity of antibodies to PfAMA1. Effective inhibition of the genetically-engineered PvAMA1 line by antibodies to PvAMA1 confirmed that the expressed PvAMA1 was functional and playing an important role in invasion. Furthermore, studies of invasion rates and kinetics suggested that the invasion of the PvAMA1 transgenic parasites appeared to be as efficient as invasion by the *P. falciparum* line that was transfected with PfAMA1 as a control, *in vitro*.

Given the substantial sequence differences between the two AMA1 species, and because AMA1 utilises its own parasite-derived protein as a receptor rather than a conserved
host molecule, we anticipated that PvAMA1 would not be able to complement the function of PfAMA1 in *P. falciparum*. However, surprisingly, we were able to generate novel chimeric *P. falciparum* with PvAMA1 replacing endogenous PfAMA1. This suggests there is substantial molecular flexibility in the receptor-binding interactions and unexpected conservation of function between species despite sequence divergence. Sufficient structural features between AMA1 species may enable a degree of cross-species conservation of function, and flexibility may be achieved through multiple binding sites for AMA1-Ron2 interactions. Indeed, recent binding studies have shown that a peptide derived from the externally exposed region of *P. vivax* Ron2 (PvRON2sp1) shows cross-reactive binding to recombinant PfAMA1 and PvAMA1 proteins [33]. This may have contributed to an environment that allowed the divergence between species of both AMA1 and its parasite-derived receptor, Ron2. Divergence may have been driven by functional adaptation, and by host immunity, since PfAMA1 and PvAMA1 are targets of acquired immunity. We believe this is the first demonstration of functional conservation of an invasion ligand between *P. falciparum* and *P. vivax* and establishes an important new paradigm.

A key difference between the two AMA1 species is that PfAMA1 has a pro-domain that is proteolytically cleaved prior to RBC invasion [27]. Key to successful replacement of the endogenous PfAMA1 with PvAMA1 in *P. falciparum* was the fusion of the pro-domain of PfAMA1 to the N-terminal sequence of PvAMA1. This suggests the pro-domain plays an important role in AMA1 expression or presentation in *P. falciparum*. [31]

Antibodies elicited by immunisation with *P. falciparum* AMA1 were cross-reactive with both native and recombinant forms of PvAMA1 using standard immunoassays (ELISA, western blotting, IFA), but either not cross-inhibitory or weakly inhibitory when measured by GIA. While antibodies elicited by immunisation with *P. vivax* AMA1 were moderately cross-reactive to *P. falciparum* AMA1 alleles using ELISA and western blotting, the functional invasion-inhibitory capacity of these antibodies was clearly *P. vivax* species-
specific. This highlights the limitations of relying on these standard immunoassays for predicting functional or protective antibodies and evaluating AMA1 vaccine responses. By generating transgenic *P. falciparum* lines that are based on the same parental line and differ only in the type of AMA1 they express, we have established valuable tools for measuring the acquisition of species-specific functional antibodies following AMA1 immunisation. These tools can now be used in future vaccine studies to identify immunogens or approaches that induce potent inhibitory antibodies targeting PvAMA1. Additionally, future studies could investigate the impact of polymorphisms in PvAMA1 on immune escape, and quantify naturally-acquired functional antibodies. There is also significant interest in development new anti-malarial drugs based on blocking PfAMA1 function, particularly its interaction with Ron2 [34] [35]. As it has recently been shown that recombinant PvAMA1 also binds to the PvRON2sp1 peptide in a similar manner [33], compounds that block this interaction may also have potential as future anti-*P. vivax* drugs.

In conclusion, malaria caused by *P. vivax* is a major global health problem, and in many parts of Asia and the Americas *P. vivax* is the predominant form of malaria. A greater understanding the molecular basis of *P. vivax* invasion, and how it diverges from *P. falciparum*, is needed to advance the development of vaccines. Our findings provide valuable new insights into the functional conservation and divergence of AMA1 in *P. vivax* and *P. falciparum*, and establish powerful new tools to dissect molecular interactions and to accelerate the development of vaccines and therapeutics based on PvAMA1. *P. falciparum* expressing PvAMA1 provide a powerful tool to evaluate functional inhibitory antibodies for vaccine development, refinement, and evaluation in a way that has not previously been possible.
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CONFLICTING INTERESTS

None of the authors have any competing interests to declare.
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FIGURE LEGENDS

Figure 1. Conserved and divergent features of *P. falciparum* and *P. vivax* AMA1. A. Phylogenetic relationship of *P. falciparum* (Pf.) AMA1 to orthologues found in other *Plasmodium* spp. Parasite species is listed in italics, host species in brackets. *Plasmodium* spp. that are transmitted through a human host are listed in red fonts B. Schematic representations of *P. falciparum* (Pf) and *P. vivax* (Pv) AMA1. The positions of amino acids (aa) that define the Pro-domain (Pro) Domain I (DI), Domain II (DII) and Domain III (DIII) of PfAMA1 [27] and PvAMA1 are shown. The extracellular Pf. ectodomain is composed of aa 25 to 546 and excludes the signal sequence (SS), Transmembrane domain (TM) and intracellular cytoplasmic tail (CT). Top numbering of PvAMA1-T defines the positions of amino acids in the PvAMA1-T hybrid protein, bottom numbering defines the positions of the same amino acids in the wild type PvAMA1 protein. Arrows define the predicted molecular weight of selected protein segments.

Figure 2. Expression and cleavage of PvAMA1 in *P. falciparum*. A. Phenotypic confirmation of PvAMA1 integration by western blot size shift. Native and reduced schizont extracts of transgenic *P. falciparum* lines expressing either W2Mef AMA1 (W2-W2) or PvAMA1 (W2-PvAMA1) were probed with anti-W2Mef AMA1 polyclonal antisera (1:1000) or 20μg/ml anti-PvAMA1 rabbit IgG (R1387 and R1388), which cross-reacted with both native and reduced forms of the 83kDa W2Mef AMA1 and the smaller 81kDa PvAMA1. Equal loading of schizont samples was confirmed by probing blots with 1:2000 anti-HSP70 antibodies.

B: Antibody binding to processed 3D7 AMA1 and PvAMA1 native proteins expressed by Compound-1 treated W2-3D7 and W2-PvAMA1 arrested schizonts by western blot.
(20μg/ml anti-3D7 AMA1 rabbit IgG, 20μg/ml anti-PvAMA1 rabbit 1387 and 1388 IgG),

Equal loading of segmented schizont samples was confirmed by probing blots with 1:2000 anti-PfRon4.2 mouse monoclonal antibodies. PfRon4.2 and Anti-3D7 AMA1 probed gels were loaded with 5μl per well of each sample. Anti-PvAMA1 probed gels were loaded with 20μl per well of each sample.

**Figure 3. Localisation of PvAMA1 in *P. falciparum.***

Apical localisation of PvAMA1 or W2Mef AMA1 in transgenic *P. falciparum* as visualised by Indirect Immuno-fluorescent microscopy (IFA).

**Figure 4. Expression of PvAMA1 in *P. falciparum* does not affect *P. falciparum* RBC invasion kinetics.** Merozoite invasion kinetics of WT W2Mef and transgenic *P. falciparum* lines expressing W2Mef AMA1 (W2-W2) or PvAMA1 (W2-PvAMA1), as measured by live cell imaging. N= the number of events filmed per stage of invasion. Statistical analysis by Mann-Whitney test (All other panels).

**Figure 5. Species-specific inhibition of invasion by antibodies to AMA1 of *P. vivax* and *P. falciparum.*** Invasion inhibition of WT W2Mef *P. falciparum* or transgenic *P. falciparum* expressing W2Mef AMA1 (W2-W2), 3D7 AMA1 (W2-3D7), FVO AMA1 (W2-FVO) or Palo Alto *P. vivax* AMA1 (W2-PvAMA1) by A. anti-PvAMA1 IgG (Rabbits 1387 and 1388) tested at 2mg/ml or 4mg/ml; B. Anti-W2Mef AMA1 sera tested at a final dilution of 1:10; C. 2mg/ml anti-3D7 AMA1 rabbit IgG; D. 2mg/ml anti-FVO AMA1 rabbit IgG. Mean and SD of two, 2-cycle growth inhibition assays performed in triplicate. Statistical analysis by Mann-Whitney Test. * (P<0.05 when compared to PvAMA1 invasion inhibition).
Figure 6. Antibodies against *P. vivax* and *P. falciparum* AMA1 cross react with native AMA1 from both species in western blot and ELISA. **A. Top panel:** Anti-PfAMA1 antibody binding to native and reduced forms of PvAMA1 present in W2-PvAMA1 schizont extracts by western blot. **Bottom panel:** Anti-PvAMA1 and anti-PfAMA1 antibody binding to refolded and reduced forms of recombinant PvAMA1 by western blot. (50ng PvAMA1 protein/well, 20µg/ml anti-PvAMA1/3D7 AMA1/FVO AMA1 rabbit IgG, 1:10 anti-W2Mef AMA1 rabbit sera, 1:2000 anti-HSP70). **B.** Anti-PfAMA1 antibody binding to recombinant PvAMA1 by ELISA. Mean and SD of each dilution performed in duplicate wells. **C-D,** Anti-PvAMA1 antibody binding to recombinant PvAMA1, W2Mef AMA1, 3D7 AMA1 or FVO AMA1 protein by ELISA. Mean and SD of each dilution performed in duplicate wells.
Figure 1

A

B. bovis (bovine)

T. gondii (feline)

P. berghei (rodent)
P. yoelii (rodent)
P. chabaudi (rodent)
P. falciparum (human)
P. reichenowi (primate)
P. galinaceum (avian)
P. relictum (avian)
P. malariae (human)
P. ovale (human)
P. coatneyi (simian)
P. knowlesi (simian)
P. cynomolgi (simian)
P. vivax (human)

0.2

B

PfAMA1

63.7 kDa

69.2 kDa

PvAMA1

53.5 kDa

PvAMA1-T

68.5 kDa
Figure 2

A

83 kd PfAMA1
66 kd PfAMA1

αHSP70
αW2MefAMA1
αPvAMA1 R1388
αPvAMA1 R1387

81 kd PvAMA1

B

180 kd PfRON4
83 kd PfAMA1
66 kd PfAMA1

αRon4.2
α3D7 AMA1
αPvAMA1 R1388
αPvAMA1 R1387

81 kd PvAMA1
64 kd PvAMA1
Figure 3
Figure 4

- First contact to start of deformation
  - W2-W2: N=7
  - W2-PvAMA1: N=9
  - P = 0.5855

- Start of deformation to end of deformation
  - W2-W2: N=7
  - W2-PvAMA1: N=9
  - P = 0.9624

- End of deformation to start of invasion
  - W2-W2: N=7
  - W2-PvAMA1: N=9
  - P = 0.1885

- Initiation of invasion to end of internalization
  - W2-W2: N=5
  - W2-PvAMA1: N=10
  - P = 0.8335
Figure 5
Figure 6

A

81 kd PvAMA1 native protein ——— W2-PvAMA1 schizont extract

60 kd PvAMA1 Ecto protein ——— Recombinant protein

B

PVAMA1 coating antigen

Polyclonal Rabbit IgG concentration

C

Rabbit 1388 Anti-PvAMA1 IgG

Polyclonal Rabbit IgG concentration

D

Rabbit 1387 Anti-PvAMA1 IgG

Coating antigen

Polyclonal Rabbit IgG concentration