Antibodies to a Single, Conserved Epitope in *Anopheles* APN1 Inhibit Universal Transmission of *Plasmodium falciparum* and *Plasmodium vivax* Malaria

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Malaria transmission-blocking vaccines (TBVs) represent a promising approach for the elimination and eradication of this disease. AnAPN1 is a lead TBV candidate that targets a surface antigen on the midgut of the obligate vector of the *Plasmodium* parasite, the *Anopheles* mosquito. In this study, we demonstrated that antibodies targeting AnAPN1 block transmission of *Plasmodium falciparum* and *Plasmodium vivax* across distantly related anopheline species in countries to which malaria is endemic. Using a biochemical and immunological approach, we determined that the mechanism of action for this phenomenon stems from antibody recognition of a single protective epitope on AnAPN1, which we found to be immunogenic in murine and nonhuman primate models and highly conserved among anophelines. These data indicate that AnAPN1 meets the established target product profile for TBVs and suggest a potential key role for an AnAPN1-based panmalaria TBV in the effort to eradicate malaria.

Malaria continues to be a tremendous public health burden worldwide, resulting in 700,000 (1) to 1.2 million deaths annually (2). With several malaria vaccines entering clinical trials (3) (PATH Malaria Vaccine Initiative Portfolio [<http://www.malariavaccine.org/rd-portfolio.php>]), the fight against this disease has entered a new era, in which elimination, and ultimately eradication, is the goal (4). To achieve this objective, malaria transmission must be interrupted by reducing the basic reproduction rate (R_0), or number of secondary cases arising from a single case, to less than one. Transmission-blocking vaccines (TBVs) are considered essential tools for meeting this goal (4–9). Malaria transmission is contingent upon successful sporogonic development of *Plasmodium* parasites in *Anopheles* mosquitoes, which begins with differentiation of male and female gametocytes into gametes, followed by mating and formation of a motile zygote, or ookinete. The ookinete must attach to, invade, and traverse the midgut epithelium to form an oocyst and undergo sporogony. Oocysts rupture after 10 to 15 days, releasing sporozoites into the hemocoel, which ultimately reach and invade the salivary glands, at which point the mosquito is infectious. TBVs elicit inhibitory antibodies against parasite sexual/mosquito stage (6, 10–13) or mosquito midgut antigens (7, 8) that when ingested by the mosquito during blood feeding on an immunized host will ultimately disrupt sporogony, arresting transmission into new human hosts.

The target product profile (TPP) indicates that the ideal malaria TBV must be immunogenic and safe across all age groups and effective against both *Plasmodium falciparum* and *Plasmodium vivax* (14). A TBV that targets a mosquito midgut antigen must additionally be highly conserved among *Anopheles* mosquitoes, of which approximately 50 of the more than 500 known species have been identified as competent vectors (15). A glycosylphosphatidyl inositol-anchored, midgut-specific alanyl aminopepti-

dase (AnAPN1) originally described for the African vector, *Anopheles gambiae*, has been found to play a critical role in ookinete invasion (16, 17). A 135-amino-acid fragment located 59 amino acids downstream of the N terminus of mature AnAPN1 (rAnAPN1^{60–195}) has been shown to be safe and highly immunogenic, even in the absence of an adjuvant, in murine models (17) and is capable of inducing antibodies in rabbits and mice that inhibit development of *P. falciparum* and *Plasmodium berghei* in *An. gambiae* and *Anopheles stephensi*, respectively, in laboratory assays (16, 17). However, laboratory assays using culture-adapted *P. falciparum* for more than 3 decades or rodent malaria parasites have proven to be poor predictors of downstream success in field trials for vaccines (18). In natural isolates from countries to which malaria is endemic, *P. falciparum* displays a wide genetic diversity and multiplicity of infection, which is not represented by the current culture-adapted strains, including the commonly used NF54 isolate and 3D7 clone (19).

To address the shortcomings in the use of laboratory assays to

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predict the potential utility of a TBV in reducing malaria transmission, we report on the findings of field-based membrane-feeding assays in two divergent malaria transmission settings (Cameroon and Thailand) to determine if the blocking efficacy observed in the laboratory, at least for *P. falciparum*, will hold true. Moreover, we extended our studies further, and we report on the results of an immunological and biochemical interrogation of the mechanism of transmission-blocking anti-AnAPN1 antibodies to better inform the current vaccine design and delivery methods and thereby further our understanding of the biology of parasite midgut invasion, particularly with respect to patent biological differences between *P. falciparum* and *P. vivax*. Small-animal and nonhuman primate (NHP) studies indicate that immunization with AnAPN1 elicits long-lasting antibodies that recognize two linear B cell epitopes, only one of which was found to be necessary and sufficient for transmission-blocking activity against *P. falciparum* and *P. vivax*. The protective epitope appears to be highly conserved among divergent *Anopheles* vector species, and although it localizes near the catalytic site of AnAPN1, antibodies directed against it do not inhibit enzymatic activity of a near-full-length recombinant AnAPN1. Taken together, the data provide significant support for the continued development of the AnAPN1 TBV and is a vital step forward in bringing this unique malaria vaccine concept to clinical trials.

MATERIALS AND METHODS

Field membrane-feeding transmission-blocking assays. In April and November 2007, *P. falciparum* gametocyte carriers (5 to 11 years old) from the Mfou district, Cameroon, were enrolled in the study upon receiving informed consent from their legal guardians. *P. vivax* gametocyte carriers (≥ 15 years old) were recruited from health clinics in Mae Sod and Kanchanaburi, Thailand, in 2007 and 2012, respectively. Informed consent was provided directly by individuals ≥ 20 years of age or was provided by the legal guardian. Infective venous blood was collected and prepared as described previously (20–22). Transmission-blocking assays were performed using rabbit anti-AnAPN1^{60–195} IgG diluted in nonimmune human AB serum or AB serum alone as a control. Total rabbit anti-AnAPN1^{60–195} IgG was purified from antisera (Washington Biotechnologies, Baltimore, MD) using Melon Gel IgG purification resin (Pierce) as described previously (16). Antibody/serum was added directly to the infective blood meal prior to feeding to mosquitoes through a membrane feeder. Total rabbit IgG dilutions of 0.1, 0.4, 0.8, 1.2, and 1.6 mg/ml were tested against *P. falciparum* parasites from a single carrier. In Mae Sod, Thailand, total rabbit IgG dilutions of 0.1, 0.4, and 1.6 mg/ml were tested against *P. vivax* parasites from a single carrier, whereas 1.6 mg/ml was tested in Kanchanaburi. Colony mosquitoes, established from field-caught populations of *An. gambiae* (Kisumu or Ngousso strain) and *Anopheles dirus* A (Bangkok) mosquitoes were used in Cameroon and Thailand, respectively. Midguts were dissected and oocysts enumerated at 7 to 8 days post-blood feeding by microscopy. The National Ethics Committee of Cameroon, the Armed Forces Research Institute for Medical Sciences-USAMC, and the Thailand Ministry of Public Health Institutional Review Boards approved human subject research experimental procedures.

Efficacy in reduction of oocyst intensity and prevalence was calculated as $[(C - E)/C] \times 100$, where C is the mean prevalence/intensity in the control group and E is the mean prevalence/intensity in the intervention group. Generalized linear mixed-effects models were used to determine the efficacies of different anti-AnAPN1^{60–195} antibody dilutions across all feeding experiments (22). Data from Cameroon and Thailand were initially analyzed independently. A binomial error structure was used for the parasite presence/absence data, while a zero-inflated negative binomial distribution was used to describe mosquito oocyst intensity. Treatment

(serum only or anti-AnAPN1^{60–195} IgG) was included as the fixed effect, while oocyst development was allowed to vary at random between replicates, reflecting different infectivities of the blood donors. Models with or without treatment information were compared using the likelihood ratio test to determine whether the intervention significantly reduced oocyst development.

AnAPN1 immunizations. Five BALB/c and 10 Swiss Webster (SW) female mice (20 to 24 g; 7 to 8 weeks old) were primed via subcutaneous injection and boosted three times at 2-week intervals via intraperitoneal (i.p.) injection with 5 μ g/ml of rAnAPN1^{60–195} in 15% sucrose–10 mM Tris–0.2% Tween 80 buffer (ST/T80) (17) emulsified (1:1) in incomplete Freund's adjuvant (IFA). Five BALB/c and five SW control mice were primed or boosted with buffer only emulsified (1:1) with IFA. Serum was collected from each mouse prior to each priming and boosting immunization. Animals were sacrificed 2 weeks following the final boosting immunization, and serum was collected via cardiac puncture. Transgenic C57BL/6 HLA-DR 2 (23), 3 (24), and 4 (25) mice (three females and three males per group) were primed and boosted (i.p.) 28 days later with 10 μ g AnAPN1^{60–195} in formulation with Alhydrogel. Control mice for each group received Alhydrogel only. Serum was collected weekly from each mouse for 6 weeks, and again via cardiac puncture when the animals were terminated 70 days postpriming. Four female *Macaca mulatta* (India strain, 11 to 13 kg) NHPs were primed and boosted (28 and 70 days postpriming) with 0.5 ml rAnAPN1^{60–195} (0.1 mg/ml) in ST/T80 buffer formulated with Alhydrogel (0.8 mg/ml) via intramuscular injection. Serum was collected on days 14, 28, 56, 70, 90, and 148 postpriming. All mouse and NHP studies were performed in accordance with Johns Hopkins University (JHU) ACUC (Animal Welfare Assurance number A3272-1) regulations. The animal protocols (MO12H232/PR11H18) were reviewed and approved by the JHU ACUC and are in compliance with U.S. Animal Welfare Act regulations and Public Health Service (PHS) policy.

AnAPN1^{60–195} indirect enzyme-linked immunosorbent assays. Serum anti-AnAPN1^{60–195} titers for each individual mouse and NHP were determined via indirect enzyme-linked immunosorbent assay (ELISA) as previously described (17). ELISAs with NHP serum were modified as follows: anti-AnAPN1^{60–195} antibodies were first probed with 100 μ l of mouse anti-monkey (*Rhesus macaque*) IgG(H+L) (Thermo Scientific) diluted 1:1,000 in blocking buffer prior to washing and detection with 100 μ l of a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG(H+L) (KPL) diluted 1:5,000 in blocking buffer. Serum endpoint titers were defined as serum dilutions giving an absorbance higher than the average optical density (OD) at 405 nm of preimmune/control serum plus three standard deviations. To measure the amount of AnAPN1^{60–195}-specific IgG present in the total rabbit IgG used in the field assays, this same ELISA platform was performed with the inclusion of a series of known concentrations of normal rabbit IgG, from which a standard curve was created to extrapolate the sample antibody concentration from the OD (data not shown). From these data, it was estimated that ~ 10 μ g/ml of AnAPN1^{60–195}-specific IgG was present at the highest concentration (1.6 mg/ml) of total rabbit IgG used in the field membrane-feeding assays.

Mouse antibody isotyping. Anti-AnAPN1^{60–195} immunoglobulin isotypes present in mouse serum (1:100, terminal bleed) were detected using a panel of rabbit anti-mouse subclass-specific antisera (Mouse Typer Isotyping Panel; Bio-Rad) by indirect ELISA, according to the manufacturer's instructions. ELISA plates were coated and blocked, and rabbit antisera were detected as described above for the AnAPN1 ELISAs. Mouse immunoglobulin isotype controls (Southern Biotech) were used as positive controls.

Determination of affinity indices. Anti-AnAPN1^{60–195} antibody affinity for AnAPN1 was determined by ELISA using thiocyanate elution. AnAPN1^{60–195} antisera from BALB/c and SW mice (1:100, terminal bleed), rabbits (1:100, terminal bleed), and NHPs (1:10, day 56 bleed) were used following the protocol described above for AnAPN1^{60–195} indirect ELISAs. Absorbance readings in the presence of sodium thiocyanate

were plotted as the $\log_{10}$ (% initial binding) versus the molar concentration of sodium thiocyanate.

SDS/PAGE and immunoblotting. *An. gambiae* midgut brush border microvillus lysates, prepared as previously described (26), were loaded (10 μ g/well) and resolved in 10% Tris-glycine gels under reducing conditions. Immunoblots were blocked with LiCor blocking buffer (Li-Cor Biosciences) diluted 1:1 in phosphate-buffered saline (PBS) for 1 h at room temperature and probed overnight at 4°C with total IgG purified from mouse, rabbit, or NHP AnAPN1^{60–195} antisera, SW mouse or NHP peptide 9-specific IgG, or total IgG purified from BALB/c mouse peptide 1, peptide 9, or keyhole limpet hemocyanin (KLH) antisera, all of which were diluted in blocking buffer plus 0.1% Tween 20 at a concentration of 10 μ g/ml. Immunoblots were washed with phosphate-buffered saline plus 0.5% Tween 20 (PBST20) and incubated with IRDye 800CW goat anti-mouse or goat anti-rabbit IgG(H+L) (Li-Cor Biosciences) diluted 1:50,000 in blocking buffer plus 0.1% Tween 20 and 0.01% SDS for 1 h at room temperature. For NHP antisera, a mouse anti-monkey antibody was used as an unlabeled secondary followed by a tertiary stain using IRDye 800CW-conjugated goat anti-mouse antibodies. Following washing, immunoblots were imaged using the Odyssey infrared imaging system (Li-Cor Biosciences).

AnAPN1 *in silico* epitope prediction. Linear B cell epitope predictions were based on physicochemical properties (27–30) of rAnAPN1^{60–195} using the Bcepred server (31) and Immune Epitope Database (IEDP) (32). A window length of seven amino acid residues was used to identify stretches of amino acids scoring above the default thresholds. The Merck Research Labs Epitope Identification software suite (U.S. patent 7756644) was used to search for potential T cell epitopes with significant sequence identity (i.e., ≥ 8 amino acids in a span of 9, identical in any human protein) with human 9-mer peptides and predict major histocompatibility complex II (MHC II) binding peptides within rAnAPN1^{60–195}.

Peptide indirect enzyme-linked immunosorbent assays. For epitope mapping studies, peptides (GenScript; American Peptide Company) were cross-linked to 96-well Maxisorp plates (Nunc, Thermo Scientific) coated with 50 μ g/ml poly-L-lysine using 0.1% glutaraldehyde in PBS. Reactive aldehyde sites were blocked with 1 M glycine, and plates were further blocked with 5% (wt/vol) nonfat milk (Bio-Rad) mixed 1:1 with 1% (wt/vol) gelatin (Bio-Rad) in PBS. Preimmune control serum from all animals and AnAPN1^{60–195} antisera from BALB/c and SW mice (1:100, terminal bleed), rabbits (1:100, terminal bleed), and NHPs (1:10, day 56 bleed) were diluted in blocking buffer and used to probe peptides prior to detection in an HRP-3,3',5,5'-tetramethylbenzidine (TMB) ELISA as described above, with an HRP-conjugated goat anti-rabbit IgG(H+L) (KPL) being used to detect rabbit anti-AnAPN1^{60–195} antibodies.

Peptide 9 depletion assays. Peptide 9-specific antibodies were purified from SW mouse and NHP total IgG by affinity chromatography using iodoacetyl coupling resin (Pierce) coupled to 1 mg of peptide 9 according to the manufacturer's instructions. Column flowthrough and washes, containing IgG depleted of peptide 9-specific antibodies, were pooled and then concentrated and exchanged into PBS buffer using Amicon Ultra centrifugal filters (50-kDa molecular-mass cutoff; Millipore). Column eluate, containing peptide 9-specific IgG, was similarly prepared. Both peptide 9-depleted and peptide 9-specific IgG were quantified by bicinchoninic acid protein assay (Pierce). The status of both IgG samples as peptide 9 depleted or peptide 9 specific was confirmed by peptide indirect ELISA as described above. Transmission-blocking activities of peptide 9-depleted and peptide 9-specific IgG were tested by standard membrane-feeding assays (SMFA) simultaneously with the total SW mouse and NHP anti-AnAPN1^{60–195} IgG from which these populations were purified.

Laboratory membrane-feeding transmission-blocking assays. *P. falciparum* (NF54) gametocyte cultures were harvested 16 to 17 days after initiation and diluted with human AB serum and red blood cells at 0.3% gametocytemia and 50% hematocrit. Infective blood was mixed with a control (IFA or KLH or preimmune), anti-AnAPN1^{60–195}, or anti-peptide IgG prior to delivery directly into water-jacketed membrane feeders

maintained at 37°C via a circulating water bath. Mouse and NHP (preimmune and immune) sera were pooled, and total IgG was purified by affinity chromatography using protein G resin (GenScript). The final concentration of IFA/preimmune control or anti-AnAPN1^{60–195} IgG in a 150- μ l total volume of infective blood was 1 mg/ml. KLH control and anti-peptide 9 IgG were used at final concentrations of 0.5 mg/ml. As expected, preimmune IgG from NHPs did not confer transmission-blocking activity compared to results with human AB serum controls (data not shown). Fifty female *An. gambiae* (Keele) mosquitoes were allowed to feed from each feeder for 30 min, after which any unfed mosquitoes were collected and discarded. Midguts were dissected, and oocysts were enumerated by microscopy 8 days post-blood feeding. Three independent replicate experiments were performed for each test antibody. Nonparametric statistical analyses (Mann-Whitney *U* test and Kruskal-Wallis one-way analysis of variance with subsequent Dunn multiple-comparison tests) were used to evaluate differences in median oocyst intensity between controls and test IgG groups.

Peptide immunizations. Five female BALB/c mice (7 weeks of age) were primed (subcutaneously [s.c.]) and boosted (i.p.) three times at 2-week intervals with 5 μ g/ml peptide 9 conjugated (1:1) with KLH in (PBS in a 1:1 emulsion with IFA. Five control mice were primed and boosted with 5 μ g KLH carrier in PBS emulsified (1:1) with IFA. Serum was collected from each individual mouse prior to each priming and boosting immunization. Animals were sacrificed 2 weeks following the final boosting immunization, and serum was collected via cardiac puncture.

Alignment of AnAPN1 homologs. *An. gambiae* AnAPN1^{60–195} and putative culicine orthologs were aligned using a combination of the MAFFT and probcons multiple sequence alignment algorithms through the T-coffee web-based server (<http://tcoffee.crg.cat/>).

AnAPN1 homology model. Homology modeling of AnAPN1 was performed using the ModWeb Comparative Modeling server (33). The model is based on the crystal structure of human aminopeptidase N (PDB no. 4FYQ) (34), which has 34% sequence identity to AnAPN1. The model spans residues 62 to 939 and was considered to be reliable based on scores for the following quality criteria: ModPipe quality score (MPQS) (1.26), TSVMod root mean square deviation (RMSD) (10.291), TVSMod NO35 (0.59) (35), GA341 (1.00), E value (0) (36), and zDOPE (−0.53). Figures were created using the software program PyMOL (<http://www.pymol.org>).

Cloning and expression of rAnAPN1^{60–942}. The near-full-length coding sequence of *An. gambiae* AnAPN1 was amplified from midgut cDNA (pMTAPN1F, 5'-TACCTACCATGGCCGCCATACAAGAGTAGTGGA-3' and pMTAPN1R 5'-GATATGGCGGCCCGCTCGGCTAGGAAGTTGGACAG-3') and cloned into the pMT/Bip/V5-His C *Drosophila melanogaster* expression vector using the NcoI and NotI restriction enzymes. *Drosophila* S2 cells were stably transfected with the expression vector using Effectene transfection reagent (Qiagen) and grown in the presence of hygromycin B (300 μ g/ml). Expression was induced upon addition of copper sulfate (600 μ M), and near-full-length recombinant AnAPN1 (rAnAPN1^{60–942}) was recovered from the supernatant 24 h later by concentration with PEG-8000 in Spectra/Por 2 dialysis sacks (12- to 14-kDa molecular-mass cutoff; Spectrum Labs) and subsequent purification using nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen).

Aminopeptidase activity assays. Aminopeptidase activity of rAnAPN1^{60–942} (1 μ g/ml) in the presence or absence of mouse and NHP peptide-9-specific or preimmune IgG (10 μ g/ml) was measured spectrophotometrically with leucine *p*-nitroaniline substrate (Sigma) by following the continuous increase in absorbance at 405 nm due to the release of 4-nitroaniline (37, 38). *Streptomyces griseus* aminopeptidase (Sigma) was used as a positive control for enzymatic activity, while the aminopeptidase inhibitor bestatin (100 μ M; Sigma) and metalloprotease inhibitor 1,10-phenanthroline (10 μ M; Sigma) were used as controls for inhibition.

FIG 1 Rabbit anti-AnAPN1⁶⁰⁻¹⁹⁵ antibodies block development of naturally circulating *P. falciparum* and *P. vivax*. Membrane-feeding assays conducted with blood collected from gametocytic volunteers in Yaoundé, Cameroon, demonstrated that rabbit anti-AnAPN1⁶⁰⁻¹⁹⁵ IgG (open circles) inhibited *P. falciparum* oocyst development in *An. gambiae* Kisumu (A and B) or Ngousso (C and D) strain mosquitoes in a dose-dependent manner compared to results with normal AB serum (filled circles) in all experiments. Rabbit anti-AnAPN1⁶⁰⁻¹⁹⁵ IgG similarly inhibited *P. vivax* oocyst development in *An. dirus* mosquitoes in Mae Sod (E) or Kanchanaburi (F), Thailand. Mosquito midguts were dissected 7 or 8 days post-blood feeding, and the number of oocysts per midgut was determined for each antibody concentration (0.1 to 1.6 mg/ml) indicated on the x axis for each gametocytic blood donor, as indicated by the codes below. Horizontal bars represent mean oocyst numbers. For each experiment, each antibody concentration that was significantly effective at reducing oocyst intensity is indicated with an asterisk.

RESULTS

AnAPN1 is essential for *P. falciparum* and *P. vivax* development in mosquitoes. We have previously shown that anti-AnAPN1⁶⁰⁻¹⁹⁵ antibodies block development of laboratory strains of *P. falciparum* (NF54) and *P. berghei* (ANKA 2.34) in *An. gambiae* and *An. stephensi* (16, 17). However, the culture-adapted NF54 strain is no longer representative of current field phenotypes (19). Therefore, we determined the transmission-blocking efficacy of anti-AnAPN1⁶⁰⁻¹⁹⁵ antibodies against naturally circulating *P. falciparum* obtained directly from individuals in Yaoundé, Cameroon, using membrane-feeding assays (MFA). Mosquito in-

fections of various intensities were achieved with blood collected from 11 volunteers with various gametocytemias over two transmission seasons (Fig. 1A to D; see also Table S1 in the supplemental material). Rabbit anti-AnAPN1⁶⁰⁻¹⁹⁵ IgG was significantly effective at reducing both the prevalence and intensity of *P. falciparum* infection in blood from each donor for the *An. gambiae* Ngousso and Kisumu strains, which represent both chromosomal forms (M and S, respectively) of this species (see Table S1), and across all donors (Table 1). Inhibition was antibody dose dependent, with complete blocking (100% efficacy) observed at the highest total IgG concentration (1.6 mg/ml), which corresponds

TABLE 1 Transmission-blocking efficacy of rabbit anti-AnAPN1^{60–195} IgG against naturally circulating *P. falciparum* and *P. vivax*

Parameter ^a	Value for infection group and IgG concn (mg/ml)							
	<i>P. falciparum</i> - <i>An. gambiae</i> infections, Cameroon					<i>P. vivax</i> - <i>An. dirus</i> infections, Thailand		
	0.1	0.4	0.8	1.2	1.6	0.1	0.4	1.6
Oocyst intensity								
Mean, control	10.16	10.16	10.13	21.68	10.16	5.97	5.97	33.51
Mean, AnAPN1	7.93	5.29	1.01	0.03	0.00	8.86	7.08	8.28
Efficacy	22.33	57.4	93.77	99.87	100	−0.17	25.9	78.02
95% CI, lower	9.8	48.4	99.59	99.59	100	−34.1	−2.1	71.49
95% CI, upper	33.12	64.82	99.96	99.96	100	25.16	46.4	83.05
<i>P</i> value	0.00	0.00	0.00	0.00	0.00	1.00	0.06	0.00
Oocyst prevalence								
Mean, control	0.76	0.76	0.77	0.92	0.76	0.75	0.75	0.85
Mean, AnAPN1	0.7	0.52	0.26	0.03	0.00	0.73	0.65	0.58
Efficacy	7.2	31.8	66.8	96.8	100	3.3	12.9	32.0
95% CI, lower	−0.15	12.58	41.94	91.99	100	−4.78	−1.45	16.05
95% CI, upper	8.28	25.39	60.24	99.41	100	11.57	18.39	40.18
<i>P</i> value	0.06	0.00	0.00	0.00	0.00	0.68	0.12	0.00

^a CI, confidence interval.

to ~10 µg/ml of AnAPN1^{60–195}-specific IgG (Table 1 and Fig. 1A to D). These results exceed those obtained with laboratory strains of *P. falciparum*, for which 85% maximal inhibition was observed (16). Efficacy (as measured by changes in oocyst intensity) did not change with increased mean oocyst intensity in control groups, which ranged from 0.79 to 35.03 oocysts per midgut.

To further investigate cross-species efficacy of anti-AnAPN1^{60–195} antibodies, we tested blocking efficacy against *P. vivax* obtained from the blood of 10 volunteers over two transmission seasons in Mae Sod and Kanchanaburi, Thailand, with *Anopheles dirus*, a major mosquito vector for both *P. falciparum* and *P. vivax* transmission. Rabbit anti-AnAPN1^{60–195} antibodies significantly reduced the prevalence and intensity of infection in individual experiments (Fig. 1 E and F; see also Table S2 in the supplemental material) and across all donors but only at the highest antibody concentration (Table 1 and Fig. 1). Efficacy varied widely (see Table S2) but remained high despite the fact that complete blocking (100%) was not achieved as we had observed for *P. falciparum* in Cameroon at an equivalent antigen-specific antibody concentration of 10 µg/ml. Immunoblot analysis confirmed antibody recognition of AnAPN1 homologs in midgut lysates from both *An. dirus* and *An. gambiae* used in these experiments (see Fig. S1). While these data suggested that rAnAPN1^{60–195} is efficacious as a TBV antigen against both *P. falciparum* and *P. vivax*, it is unclear if the observed differences in transmission-blocking efficacy can be attributed to subtle variations or polymorphisms in AnAPN1^{60–195} among vectors or differences in parasite midgut invasion strategies. Unfortunately, *P. vivax* culture is currently untenable, requiring direct acquisition from infected individuals, thereby preventing a straightforward examination of these issues.

rAnAPN1 is immunogenic in multiple animal models. The observation that anti-AnAPN1^{60–195} antibodies were more effective against *P. falciparum* than against *P. vivax* stimulated an immunological, biochemical, and molecular interrogation of the transmission-blocking mechanisms of these antibodies. We first sought to more thoroughly characterize the humoral response to research-grade rAnAPN1^{60–195} produced under current good laboratory practices (cGLP) in multiple animal models. We assessed

immunogenicity and antibody affinity and performed epitope mapping studies for BALB/c and Swiss Webster (SW) mice, rabbits, and nonhuman primates (NHPs). Mice were primed with rAnAPN1^{60–195} emulsified with incomplete Freund’s adjuvant (IFA) and boosted 3 times at 2-week intervals. While we anticipated variability in the immune response, we detected high levels of rAnAPN1^{60–195}-specific IgG in the sera of all animals. When the study was terminated at day 56 postpriming, titers of antibody had only just begun to wane in BALB/c mice (Fig. 2A) but had yet to decline in SW mice (Fig. 2B). Mice achieved reciprocal serum endpoint antibody titers of 10⁵ to 10⁷ (see Fig. S2A and B in the supplemental material), exceeding what was previously reported for this antigen when using alum as an adjuvant (17). IgG1 dominated immunoglobulin profiles for BALB/c and SW mice, with contributions from IgG_{2a} and IgG_{2b} (see Fig. S3), suggesting that immunization with rAnAPN1 may induce a mixed Th1 and Th2 response.

As rAnAPN1^{60–195} progresses toward phase I clinical testing in humans, characterizing the humoral response to immunization in NHPs becomes increasingly important, providing critical insight regarding the potency and duration, as well as mechanism of action, of the anti-AnAPN1^{60–195} antibody response. Four female NHPs (*Macaca mulatta*) were primed with rAnAPN1^{60–195} completely adsorbed to the highly safe adjuvant, alum (Alhydrogel, Brenntag Biosector), and boosted 28 and 70 days later. We observed high levels of rAnAPN1^{60–195}-specific IgG, achieving serum endpoint titers >1:10⁶ at day 90 (Fig. 2C) prior to decline (see Fig. S2C in the supplemental material), in the serum of all animals as determined by ELISA throughout the 150-day study, with little variability observed among animals (Fig. 2C) and no reported reactogenicity at the inoculation site.

We investigated the immunological aspects underlying the specificity of anti-AnAPN1^{60–195} antibodies by comparing the average affinity index (AI), defined as the molar concentration of a chaotropic agent required to elute 50% of antibody from antigen in an ELISA, which revealed that mouse anti-AnAPN1^{60–195} antibodies had the strongest affinity for rAnAPN1^{60–195} (AI = 2.5), followed by those from rabbits (AI = 1.96), and NHPs (AI =

FIG 2 Immunization with rAnAPN1⁶⁰⁻¹⁹⁵ elicits a potent, long-lasting antibody response in multiple animal models. Titers of antigen-specific antibody detected by ELISA in the serum (diluted 1:100) of individual BALB/c (A) or Swiss Webster (B) mice immunized with rAnAPN1⁶⁰⁻¹⁹⁵-IFA or IFA only or NHPs (C) immunized with rAnAPN1⁶⁰⁻¹⁹⁵-Alhydrogel at each time point during the study, as indicated on the x axis. Mean absorbances $\pm$ 1 standard deviation at 450 nm from triplicate wells are plotted. Each line represents an individual animal. (D) Native AnAPN1 present in *An. gambiae* brush border microvilli (AgBBMV) protein lysates (10 μ g/lane) is recognized by total IgG (10 μ g/ml) purified from BALB/c (lane 1), SWP1 (lane 2), and SWP1+P9 (lane 3) mice and NHP (lane 4) rAnAPN1⁶⁰⁻¹⁹⁵ antisera by SDS-PAGE and Western blotting. When probed with peptide 9-specific antibodies (10 μ g/ml) purified from SWP1+P9 (SW; lane 5) or NHP (NHP; lane 6) total anti-rAnAPN1⁶⁰⁻¹⁹⁵ IgG, the same protein-banding pattern was observed.

0.72). However, equivalent concentrations of anti-AnAPN1⁶⁰⁻¹⁹⁵ IgG purified from the serum of all animals recognized cognate, native AnAPN1 from *An. gambiae* by immunoblot analysis (Fig. 2D) and significantly inhibited development of cultured *P. falciparum* (NF54) in *An. gambiae* in standard membrane-feeding assays (SMFA) (Fig. 3; see also Table S3 in the supplemental mate-

rial), suggesting a moderate affinity threshold for bioactivity had been met.

Recognition of a single linear B cell epitope on AnAPN1 confers transmission-blocking activity. To further characterize the antibody-antigen interaction, we used multiple, complementary *in silico* methods to predict linear B cell and CD4⁺ T cell epitopes within rAnAPN1⁶⁰⁻¹⁹⁵. Physicochemical properties of AnAPN1⁶⁰⁻¹⁹⁵ indicate six regions that are likely to contain linear B cell epitopes, and data generated using Epitope Identification Suite software (Merck Research Labs) predicted that several peptides within AnAPN1⁶⁰⁻¹⁹⁵ would bind strongly MHC II encoded by a variety of HLA-DRB1 alleles, including those most commonly observed among Caucasian (DRB1*04 and DRB1*08) and sub-Saharan African (DRB1*03, DRB1*11, DRB1*13, and DRB1*15) populations (39) (Fig. 4A).

We examined the potential T cell helper response to our antigen among HLA-DR allele transgenic C57/BL/6 mice, which express human MHC II, immunized with rAnAPN1⁶⁰⁻¹⁹⁵ formulated with Alhydrogel in a prime-plus-single-boost regimen, and found that it was immunogenic in male and female HLA-DR2 (human DRB1*1501) (23), HLA-DR3 (human DRB1*0301) (24), and HLA-DR4 (human DRB1*0401) (25) transgenic mice, with serum endpoint titers persisting to 70 days postpriming (Fig. 4B to D). The Epitope Identification Suite was also used to search for potential T cell epitopes within AnAPN1⁶⁰⁻¹⁹⁵ with significant homology to any human proteins ($\geq$ 8 amino acids in a span of 9). This analysis indicated that based on the currently annotated human proteome, AnAPN1⁶⁰⁻¹⁹⁵ does not share potential T cell epitopes with human sequences. When these findings are considered in combination with the observation that anti-AnAPN1⁶⁰⁻¹⁹⁵ IgG does not stain human tissues (17), these data suggest a promising safety profile for rAnAPN1⁶⁰⁻¹⁹⁵, an immunogen unlikely to cross-react with human autoantigens, and predict the possibility of functional T cell-B cell interactions in immunized human populations to drive B cell activation and antibody production. However, it should be noted that the HLA-DR3 transgenic mice express human DRB1*0301 on a mouse endogenous class II-negative background that is limited to CD4⁺ T cells, with normal expression of MHC II in spleen, lymph nodes, and peripheral blood lymphocytes, while the HLA-DR2 and HLA-DR4 mice ex-

FIG 3 Anti-AnAPN1⁶⁰⁻¹⁹⁵ antibody recognition of a single, linear B cell epitope confers transmission-blocking efficacy. Standard membrane-feeding assays were performed to assess the transmission-blocking efficacy of BALB/c (A) or Swiss Webster (B) mouse (according to epitope profile, SWP1 or SWP1+P9) or NHP (C) anti-AnAPN1⁶⁰⁻¹⁹⁵ antibodies (open circles) against *P. falciparum* (NF54) in *An. gambiae* mosquitoes. Enumeration of oocysts per midgut determined 8 days post-blood feeding revealed that oocyst intensity and prevalence were reduced compared to those for the control (IFA) IgG (filled circles) only by anti-AnAPN1⁶⁰⁻¹⁹⁵ antibodies recognizing peptide 9. This inhibition was abrogated following depletion of peptide 9-specific antibodies from SW(P1+P9) (B) or NHP (C) anti-AnAPN1⁶⁰⁻¹⁹⁵ IgG (blue circles) and recovered when feeding only peptide 9-specific IgG.

FIG 4 Anti-AnAPN1^{60–195} antibodies recognize predicted linear B cell and CD4⁺ T cell epitopes. (A) *In silico* methods utilizing physicochemical properties predict multiple linear B cell epitopes (dashed lines) within AnAPN1^{60–195}, while data generated by Epitope Identification Suite (Merck Research Labs) predict that several peptides will strongly bind MHC II encoded by a variety of DRB1 alleles (colored lines) represented among Caucasian and East African populations (DRB1*0301, DRB1*0701, and DRB1*1501). Nine peptides capturing these potential immunogenic regions of AnAPN1^{60–195} were synthesized for epitope mapping studies (solid black lines). (B to D) rAnAPN1^{60–195} elicits a strong, long-lasting humoral response in human HLA-DR2 (B), HLA-DR3 (C), or HLA-DR4 (D) transgenic C57BL/6 mice immunized with rAnAPN1^{60–195}-Alhydrogel (black lines) or Alhydrogel only (control; blue lines), as determined by ELISA. Pooled serum titers (day 70 post-priming immunization) for male (open circles) and female (filled circles) mice are plotted. Optical density (O.D. 450) and reciprocal serum dilutions are plotted. Error bars indicate ± 1 standard deviation from results for triplicate wells. (E to G) Two predominant peptides, indicated on the x axis, are recognized by anti-AnAPN1^{60–195} antibodies in the serum of BALB/c and Swiss Webster mice (E), rabbits (F), or NHPs (G) by ELISA. Mean ± 1 standard deviation absorbance readings (O.D. 450) from triplicate wells are plotted. ELISA results for an individual animal that is representative of the epitope profiles observed for each host species are shown for AnAPN1^{60–195} and preimmune (control) serum.

press chimeric class II molecules, expressing both mouse and human HLA-DR alleles.

To identify the functional linear B cell epitopes within AnAPN1^{60–195}, nine peptides capturing the predicted B and T cell epitopes were synthesized (Fig. 4 E and F; see also Table S4 in the supplemental material) and probed with individual mouse, rabbit, and NHP AnAPN1 antisera by ELISA. Two predominant epitopes within AnAPN1^{60–195} were recognized: N-terminal peptide 1 (P1) and C-terminal peptide 9 (P9). Sera from all BALB/c mice and NHPs recognized both peptides, while serum from SW mice recognized both peptides 1 and 9 (SWP1+P9) or only peptide 1 (SWP1) (Fig. 4 E to G). Surprisingly, rabbit anti-AnAPN1^{60–195} antibodies, which were used in our field studies, recognized only peptide 9 (Fig. 4 F).

Serum from SW mice was pooled by epitope profile for further studies. Comparative immunoblotting showed that IgG from

both SWP1 and SWP1+P9 mice strongly bound native midgut AnAPN1 (Fig. 2D, lanes 2 and 3), with no discernible differences in immunoglobulin isotype or affinity for rAnAPN1^{60–195}. However, development of *P. falciparum* (NF54) in *An. gambiae* was only significantly inhibited by SW mouse anti-AnAPN1^{60–195} antibodies that recognized peptide 9 in SMFAs (Fig. 3B; see also Table S3 in the supplemental material). When peptide 9-specific antibodies were depleted from SWP1+P9 and NHP anti-AnAPN1^{60–195} IgG, the transmission-blocking effect was abrogated (Fig. 3 B and C; see also Table S3). Purified mouse and NHP peptide 9-specific antibodies recognized native midgut AnAPN1 by immunoblot analysis (Fig. 2D, lanes 5 and 6) and achieved levels of inhibition similar to those observed with anti-AnAPN1^{60–195} (SWP1+P9) IgG by SMFA. Purification of peptide 9-specific antibodies revealed ~ 10 μ g per 1 mg total IgG for both mice and NHPs; however, differences in affinity for native

FIG 5 Anti-peptide 9 antibodies recognize AnAPN1 and inhibit development of *P. falciparum*. (A and B) Antigen-specific-antibody titers from serum pooled (day 56 post-priming immunization, diluted 1:100) from BALB/c mice immunized with peptide 1 (A) or 9 (B) conjugated to KLH following priming and three boosts, as determined by ELISA. Serum titers for control mice (immunized with KLH) are also plotted. Data represent serum pooled from 5 mice. Optical density (O.D. 450) and reciprocal serum dilutions are plotted. Error bars indicate ± 1 standard deviation from triplicate wells. (C) Both anti-peptide 1 (P1) and anti-peptide 9 (P9) antibodies (10 μ g/ml) bind rAnAPN1^{60–195} (0.5 μ g/ml), but only anti-peptide 9 antibodies recognize native midgut AnAPN1 in *An. gambiae* brush border microvilli (AgBBMV) lysates by Western blotting. Control anti-KLH antibodies did not bind either rAnAPN1^{60–195} or AgBBMVs. (D) Total IgG (10 μ g/ml) purified from sera of BALB/c mice immunized with peptide 9 (P9) but not peptide 1 (P1) conjugated to KLH (open circles) inhibited *P. falciparum* oocyst intensity and prevalence in *An. gambiae* compared to that of control IgG (KLH only; filled circles) in SMFAs. Horizontal bars represent mean oocyst numbers. Data in each panel represent a typical experiment utilizing IgG purified from pooled serum run in triplicate.

midgut AnAPN1 likely attributed to the observed differences in band intensity, which is supported by the affinity index data.

To confirm these results, we initiated a new study, in which BALB/c mice were immunized with peptide 1 or peptide 9 conjugated to keyhole limpet hemocyanin (KLH) in formulation with IFA. Both peptides were found to be immunogenic (Fig. 5A and B); however, while both anti-peptide 1 and anti-peptide 9 antibodies bound rAnAPN1^{60–195} as determined by ELISA and immunoblotting (Fig. 5C), only anti-peptide 9 antibodies stained native midgut AnAPN1 in immunoblots (Fig. 5C). Finally, results from SMFAs conducted with anti-peptide 9 but not anti-peptide 1 IgG mirrored those observed in the SW mouse and NHP peptide 9 depletion assays (Fig. 5D; see also Table S3 in the supplemental material).

The AnAPN1 transmission-blocking epitope is highly conserved. To evaluate the molecular diversity of AnAPN1^{60–195} and the transmission-blocking epitope (peptide 9), amino acid sequences of culicine orthologs were derived from existing genomic, transcriptomic, and proteomic data sets. AnAPN1^{60–195} orthologs were identified for anopheline vectors of human malaria, including *Anopheles albimanus* (Latin America) (40, 41), *Anopheles darlingi* (South America) (42), *Anopheles funestus* (Sub-Saharan Africa) (43, 44), and *An. gambiae* (45), and the avian malaria vectors *Aedes aegypti* (46) and *Culex quinquefasciatus* (47). An amino acid alignment of these orthologs revealed that AnAPN1^{60–195} is not only highly conserved among divergent anophelines, but a high

level of identity appears to be maintained across culicines in general. More striking is the near-100% identity at the amino acid level of peptide 9 that was observed across all *Anopheles* species (Fig. 6A). Although only lab strains of four anopheline species were included in the alignment, it is noteworthy that two subgenera (*Nyssorhynchus* and *Cellia*) are represented, which last shared a common ancestor ~ 80 million years ago (48). Given this degree of conservation between distantly related species, we find it unlikely that peptide 9 will vary at the amino acid level within species sampled from the field.

Anti-AnAPN1^{60–195} antibodies do not inhibit enzymatic activity of rAnAPN1^{60–942}. Fine-scale, epitope mapping of peptide 9 with a series of four overlapping 10- and 12-mer peptides (see Fig. S4A in the supplemental material) revealed that rabbit and mouse anti-AnAPN1^{60–195} antibodies bind two different regions of peptide 9, with NHP serum containing populations of anti-AnAPN1^{60–195} antibodies recognizing both epitopes (see Fig. S4B to D). When viewed in concert with our field-based MFA data, simply targeting peptide 9 is sufficient to convey transmission-blocking functionality. To further investigate this, a homology model of near-full-length AnAPN1 (beginning at residue 62) was derived from the human aminopeptidase N (CD13) (34) (Fig. 6B and C). Using this model, peptide 9 was predicted to localize near the binding pocket and catalytic site of AnAPN1, suggesting that anti-AnAPN1^{60–195} antibodies may possibly function by inhibiting aminopeptidase activity that may otherwise proteolytically ac-

FIG 6 Antibodies targeting the highly conserved protective epitope do not inhibit AnAPN1 aminopeptidase activity. (A) Multiple sequence alignment of the *An. gambiae* (*An. gam*) AnAPN1⁶⁰⁻¹⁹⁵ antigen with putative orthologs in *An. funestus* (*An. fun*), *An. darlingi* (*An. dar*), and *An. albimanus* (*An. alb*), *Ae. aegypti* (*Ae. aeg*), and *C. quinquefasciatus* (*Cx. qui*) reveals high conservation of AnAPN1⁶⁰⁻¹⁹⁵ and the transmission-blocking epitope, peptide 9 (light blue highlights conserved identities among 4 species, dark blue among >4 species). (B and C) Ribbon (B) and space-filling (C) homology models of AnAPN1 based on the crystal structure of human aminopeptidase N suggest that peptide 9 localizes near the binding pocket and catalytic site of AnAPN1. (D) However, mouse and NHP peptide 9-specific antibodies do not inhibit aminopeptidase activity of near-full-length recombinant AnAPN1 (rAnAPN1⁶⁰⁻⁹⁴²) expressed in *Drosophila* S2 cells compared to results for preimmune IgG in enzymatic assays utilizing an L-leucine *p*-nitroanilide substrate. The rate of rAnAPN1⁶⁰⁻⁹⁴² activity was measured as nmol *p*-nitroaniline (mean $\pm$ 1 standard deviation) formed per min at 405 nm. Bestatin and 1,10-phenanthroline were used as controls for inhibition of aminopeptidase and metalloprotease activities, respectively.

tivate a secreted ookinete invasion molecule. However, aminopeptidase activity assays demonstrated that the presence of SW mouse and NHP anti-peptide 9 antibodies had no effect on recombinant near-full-length AnAPN1 (rAnAPN1⁶⁰⁻⁹⁴²) (Fig. 6D). These data suggest that anti-AnAPN1⁶⁰⁻¹⁹⁵ antibodies act by interfering, either directly or indirectly, with an AnAPN1-ookinete protein binding interaction.

DISCUSSION

The paradigm shift toward elimination and eradication of malaria calls for new tools, including TBVs that must meet a strict TPP (4). We found that AnAPN1⁶⁰⁻¹⁹⁵ is a highly conserved anopheline mosquito midgut molecule that is critical for *P. falciparum* and *P. vivax* ookinete invasion. Immunization of NHPs with the expected clinical formulation/dose of rAnAPN1⁶⁰⁻¹⁹⁵ with Alhydrogel elicited a potent and functional humoral response that was maintained for at least 5 months, without any apparent negative immunization-related health outcomes. With a limited number of potent adjuvants available and no natural boosting, the robust, sustainable immune response to rAnAPN1⁶⁰⁻¹⁹⁵-Alhydrogel is a hallmark achievement for TBVs and malaria vaccines in general. In addition, our investigation of the mechanisms underpinning the functionality of anti-AnAPN1⁶⁰⁻¹⁹⁵ antibodies not only supports progression of the AnAPN1 TBV to phase I clinical trials but also provides new insight into the biology of falciparum/vivax-

vector interactions and has important implications for AnAPN1 TBV design.

Transmission-blocking activity of anti-AnAPN1⁶⁰⁻¹⁹⁵ antibodies against *P. falciparum* and *P. vivax* was found to be conferred through recognition of a single, highly conserved epitope. Field trials comparing the efficacies of anti-AnAPN1⁶⁰⁻¹⁹⁵ antibodies in additional *Plasmodium-Anopheles* infection models, particularly with vector species that are dually competent for both *P. falciparum* and *P. vivax* (i.e., *An. dirus* and *Anopheles farauti*), and further characterization of antibody-antigen kinetics in these species will be required to confirm this finding. Modification of the existing AnAPN1 antigen to enhance, if not focus, the immune response toward this epitope to improve vaccine efficacy is currently being evaluated. The identified transmission-blocking epitope may also provide the basis for evaluating functionality of future iterations of this AnAPN1⁶⁰⁻¹⁹⁵ protein-in-adjuvant vaccine as well as other vaccine platforms. This will be particularly salient when the full-length antigen is expressed in heterologous systems, where improper folding of the protein in addition to the presence of transmission-irrelevant but immunodominant epitopes could potentially obscure the transmission-blocking epitope and dilute the production of functional antibodies. We assume that this is likely the case for the recently reported adenovirus-vectored and wheat germ cell-free expressed AnAPN1 vac-

cines in which inhibition of *P. falciparum* was negligible (49, 50). Unlike the near-full-length AnAPN1 antigen described in this study, wheat germ cell-free expressed AnAPN1 (50), which was originally provided for our work, completely lacks enzymatic activity (data not shown).

We observed that although effective against both human malaria parasites, anti-AnAPN1^{60–195} antibodies were clearly more efficacious against *P. falciparum* than *P. vivax*, at least at the concentrations tested. Given our data, it seems unlikely that this disparity is attributed to differences in antibody-antigen interactions resulting from variations or polymorphisms of AnAPN1^{60–195} among vector species. Rather, the observed species-specific differences in anti-AnAPN1^{60–195} antibody efficacy likely stem from modifications in the ookinete midgut invasion strategy, including the sequestration of accessory midgut surface molecules at the invasion site (51) and the parasite “invasin” repertoire themselves (52), which intrinsically partition *P. falciparum* and *P. vivax*. Our data suggest that AnAPN1 enzymatic activity is not a mediator of ookinete invasion of the midgut. However, despite repeated *in situ* binding studies, near-full-length aglycosylated rAnAPN1^{60–942} did not stain the *Plasmodium* ookinete surface or microneme. These data suggest that an AnAPN1-ookinete interaction may be dependent on the conformation of a secreted ookinete micronemal protein(s) that would occur only upon direct contact with the surface of midgut microvilli. We had previously identified AnAPN1 by virtue of its C-terminal O-linked glycans (16), which are absent from near-full-length rAnAPN1^{60–942}. Thus, we hypothesize that binding to AnAPN1 *in vivo* may require the initial interaction of an ookinete lectin-like molecule with preference for O-linked glycans (53–56). Last, it has been shown that several ookinete-interacting proteins are present on the surface of the mosquito midgut epithelial microvilli (41, 51) and that AnAPN1 interaction with the ookinete may require the coordination of several other mosquito midgut molecules. Ultimately, targeting multiple midgut ligand targets or mosquito midgut and parasite antigens may be required to achieve complete TBV-mediated immunity in regions where *P. vivax* is endemic (57). Although a number of important ookinete and midgut targets have been identified, few parasite-vector interactions between these molecules have been clearly defined, and there is an urgent need for additional discovery research to identify new potential TBV targets (14).

In conclusion, rAnAPN1^{60–195} is an inherently immunogenic antigen with a promising safety profile that induces potent, long-lasting transmission-blocking antibodies in mice and NHPs. Anti-AnAPN1^{60–195} antibodies are effective against naturally circulating *P. falciparum* and *P. vivax* parasites across divergent species of anophelines through recognition of a highly conserved linear B cell and T cell epitope. Together with our previous preclinical data on antigen production, safety, potency, and efficacy (16, 17), this body of evidence indicates that AnAPN1 has the capacity to meet the established TPP for TBVs and suggests a critical role for an AnAPN1-based panmalaria TBV in the effort to eradicate malaria.

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