Dissertation

Adeno-associated virus is an effective malaria booster vaccine vector following adenovirus priming

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SUMMARY

An ideal malaria vaccine platform should potently induce protective immune responses and block parasite transmission from mosquito to human, and it should maintain these effects for an extended period. Here, we have focused on vaccine development based on adeno-associated virus serotype 1 (AAV1), a viral vector widely studied in the field of clinical gene therapy that is able to induce long-term transgene expression without causing toxicity *in vivo*. We generated a series of recombinant AAV1s and human Adenovirus type 5 (AdHu5) expressing either *Plasmodium falciparum* circumsporozoite protein (PfCSP) or P25 (Pfs25) protein, and demonstrated the potential utility of AAV1 vectors as an extremely potent booster vaccine to induce long-lasting immunity when combined with an Ad-priming vaccine in a rodent malaria model. In addition, we generated a multi-stage vaccine targeting both antigens, as it will greatly reduce the cost of administration of several single-target vaccines necessary to achieve reductions in the disease burden and transmission.

Here I show that heterologous two-dose vaccination with AdHu5-prime and AAV1-boost (AdHu5-AAV1) elicited robust and long-lasting PfCSP- or Pfs25-specific antibody over 280 days. Regarding its protective efficacy, AdHu5-AAV1 PfCSP achieved high sterile protection (up to 80% protection rate) against challenge by transgenic *Plasmodium berghei* sporozoites expressing PfCSP (*Pb*PfCSP). Regarding its transmission-blocking (TB) efficacy, immunization with AdHu5-AAV1 Pfs25 maintained TB activity *in vivo* against transgenic *P. berghei* expressing Pfs25 (*Pb*Pfs25DR3) for 287 days (99% reduction in oocyst intensity; TRA and 85% reduction in oocyst prevalence; TBA). For the multi-stage vaccines, sustained high titer of antigen-specific antibodies were also elicited in mice. In addition, complete protection was obtained after intravenous sporozoite challenges and a long-term transmission-blocking (99 % of TRA and 81% of TBA). The protection and TRA level are not significantly different compared with the mixture of single-antigen vaccines.

Our data indicate that AAV1-based malaria vaccines can confer potent and durable protection as well as TB efficacy when administered following a AdHu5-priming vaccine, supporting the further evaluation of this regimen in clinical trials as a next-generation malaria vaccine platform. I propose that the multi-stage malaria vaccine regimen will be a powerful tool for malaria eradication while providing a greater overall protection and cost-effectiveness than single-target vaccines.

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ABBREVIATIONS

AAV	Adeno-associated virus
AAV1	Adeno-associated virus serotype 1
Ab	Ab
Ad	Adenovirus
AdHu5	human adenovirus type 5
Ag	antigen
BV	baculovirus
CSP	cirucumsporozoite protein
DAPI	4′,6-diamidino-2-phenylindole
DFA	direct-feeding assay
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-liked immunospot assay
EPA	*Pseudomonas aeruginosa* exoprotein A
GLURP	glutamate-rich protein
GPI	glycosylphosphatidylinositol
HEK	human embryonic kidney
HIV	human immunodeficiency virus
HRP	horse-radish peroxidase
ICS	intracellular cytokine staining
IFA	immunofluorescence
IFN	interferon
IgG	immunoglobulin G
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
IVIS	*in vivo* imaging system
kDa	kilodaltons (protein size)
mAb	monoclonal Ab
MOI	multiplicity of infection
NAb	neutralizing Ab
NGS	normal goat serum

PBS	phosphate-buffered saline
PBST	PBS containing 0.1% tween-20
pCAG	cytomegalovirus (CMV) enhancer fused to the chicken beta-actin promoter
pCMV	cytomegalovirus immediate early promoter
PE	pre-erythrocytic
PEV	pre-erythrocytic vaccine
Pf	*Plasmodium falciparum*
PfCSP	*P falciparum* circumsporozoite
PfCSP-Tc/Pb	transgenic *P. berghei* ANKA parasite line that express *P. falciparum* CSP under the control of the *P. berghei* CSP promoter
Pfs25	*P falciparum* P25 protein
Pfs25DR3	transgenic *P. berghei* ANKA parasite line that express *P. falciparum* P25 antigen
PFU	plaque-forming unit
p.i.	post-infection
pRBC	parasitized red blood cell
ROI	region of interest
RLU	relative luminescence units
SD	Standard deviation
SEM	standard error mean
SFU	spot-forming unit
SMFA	standard membrane-feeding assay
Spz	sporozoites
TB	transmission-blocking
TBA	transmisson-blocking activity
TBV	transmission-blocking vaccines
TRA	transmission-reducing activity
vg	viral genomes
VIP	vectored immunoprophylaxis
VSV-G	vesicular stomatitis virus G glycoprotein
WGCF	wheat germ cell-free
WHO	World Health Organization
WT	wild type

INTRODUCTION

In response to the threat posed by emerging resistance to artemisinin-based chemotherapy and insecticide-treated bed nets, efforts to develop potent malaria vaccines as a complementary tool in eradicating the disease have recently been intensified (1). The most advanced *Plasmodium falciparum* malaria vaccine candidate, RTS,S/AS01 (also known as Mosquirix™), based on pre-erythrocytic stage targeting the *P. falciparum* circumsporozoite protein (PfCSP), showed a limited vaccine efficacy of 36.3% against clinical malaria among children aged 5 to 17 months and of 25.9% in young infants aged 6 to 12 weeks, which declined to 4.4% over 7 years of follow-up in phase III clinical trials in several sub-Saharan countries (2, 3). Besides its limited and short-term efficacy, RTS,S raised some safety concerns and practical deployment challenges with its four-dose regimen in target age groups at high risk of malaria (4). Because of the moderate efficacy of the RTS,S vaccine, the malaria vaccine technology roadmap has updated their strategic goals from the development of vaccines with 80% protective efficacy against *P. falciparum* by 2020 to the development of second-generation malaria vaccines for malaria elimination in multiple settings that are highly efficacious against the disease by 2030 (1, 5). Achieving this goal would require new interventions of vaccine development to complement current control strategies, such as the development of transmission-blocking (TB) vaccines (TBVs).

Malaria in human is caused by *Plasmodium spp* parasites which undergo a complex life cycle (6). The parasites are transmitted by *Anopheles* mosquitoes by injection of sporozoites during bloodmeal into the subcutaneous tissue from which they migrate to the liver and invade hepatocytes, mature to a schizont containing merozoites that invade red blood cells to commence the erythrocytic cycle. Some merozoites differentiate into gametocytes which are ingested by the female mosquitoes, recombining into ookinetes that develop into oocysts in the mosquito midgut containing a large number of sporozoites migrating to salivary gland to repeat the cycle in the next host. The antigenic characteristics of the parasites change throughout the life cycle and most antigens (Ag) are not expressed at all stages of the parasite's life cycle. Consequently malaria vaccines have been developed by targeting different stages ; pre-erythrocytic stage, erythrocytic stage, and sexual stage (7). Pre-erythrocytic (PE) stage is a prime target for intervention efforts because immunity against this stage would be sterilizing by preventing sporozoites from invading hepatocytes or liver-stage parasites from developing to maturity and releasing infective merozoites, thus

eventually preclude the development of disease and the transmission of malaria (7, 8). This stage has been the target of RTS,S/AS01 which acts through the induction of high levels of both anti-circumsporozoite (CSP) antibodies (Ab) and CSP- specific $CD4_+$ T cells, with the Ab response having a greater role (9). In recent years, there has been an increased focus on the development of vaccines to break the cycle of *Plasmodium* by targeting the sexual stages (TBV), some of which has entered clinical trials (1, 10, 11). Both vaccines, the PE and TBV, are categorized as vaccines that interrupt malaria transmission (VIMT) to support malaria elimination (12). Hence, malaria vaccines have been considered amongst the most important modalities for potential prevention of the disease and transmission reduction.

TBVs targeting the Ag expressed in the sexual stages of malarial parasites are considered one promising strategy for blocking transmission of the parasite from mosquitoes to humans. However, one potential limitation of TBVs is limited activity as the specific Ab (Ab) against the Ag, particularly with the mosquito-stage Ag Pfs25, cannot be boosted by natural infection so the titer gradually falls over time (13, 14). Hence, the development of a TBV capable of inducing long-term TB immunity for at least one transmission season (~6 months), in addition to effective pre-erythrocytic vaccines (PEVs), would be an advantageous strategy (13).

In 2013, the malaria vaccine technology roadmap set two strategic goals to be met by 2030; namely, vaccines that are highly efficacious in preventing clinical malaria and vaccines that prevent transmission to accelerate malaria parasite elimination (1). An efficacious vaccine must either be completely effective against a stage, by eliminating the parasite or dramatically reducing parasite numbers, or else be targeted at multiple stages of the parasite's life cycle (7). Since such an effective vaccine has not been available yet, combining partially effective vaccines of different anti-parasitic classes is a powerful way to achieve the goals. It has been demonstrated by a recent study that partially efficacious interventions targeting the pre-erythrocytic and the sexual stage have synergistic impact in eliminating malaria from a population over multiple generations (15). Several studies have investigated the application of a mixture or co-administration of vaccines targeting different stages (16-20) including the most recent combination of RTS,S/AS01 and Pfs25-IMX313, the most leading candidate of transmission-blocking vaccines (21) with some promising results.

In any case, a multi-stage vaccine will provide a more cost-effective solution than vaccinating with mixtures of multiple single-stage vaccines. Also, it will be more convenient than co-administration of multiple vaccines for people who get vaccinated. Unfortunately, development of such vaccine has been a minority (10). Several studies investigating the

potential of multi-stage malaria vaccines have demonstrated generally poor Ab responses and limited efficacies (22-25). However, a chimeric of *P falciparum* (Pf) glutamate-rich protein (GLURP), an Ag expressed in all stages of parasites (26), fused in frame to a correctly folded fragment of Pfs48/45, a sexual stage Ag, have shown induction of specific individual antibodies with transmission blocking activity in the standard membrane feeding assay (SMFA) and functional activity against asexual stages in the Ab-dependent cellular inhibition assay (27), denoting a potential of development of multi-stage malaria vaccines.

The progress of viral vectored vaccines for malaria through the clinical development pathway has accelerated considerably (28). Adeno-associated virus (AAV), one of widely used viral vector, is a member of the family *Parvoviridae*. It can infect a wide variety of human and nonhuman cells and is not associated with any known disease or adverse clinical effects. A key advantage of AAV vectors is their capability to mediate long-term transgene expression without causing toxicity *in vivo* (29, 30). These safety and durability profiles have made AAV an attractive vector for gene therapy; it has been tested in around 100 clinical trials (31, 32). Recently, AAV vectors have also emerged as the frontrunner in vectored immunoprophylaxis (VIP), an active approach to substitute passive immunization by facilitating the secretion of neutralizing Abs (NAbs) by human cells as AAV delivers genes encoding the NAb (33, 34). Intramuscular (i.m.) injection of VIP vectors in mice and macaques elicits long-lived Ab or Ab-related immunoadhesin production at levels sufficient to protect against HIV, simian immunodeficiency virus, and influenza A virus infection (35-38). Since both VIP and gene therapy rely on the low immunogenicity of AAV to permit durable expression of the transgene, utilization of AAV in the field of vaccination is minimal (39). Of its few investigated applications, an AAV-based malaria blood-stage vaccine has been developed but did not confer any protection against malaria parasite challenge in a mouse model when being used as a single vaccine regimen or as a booster following a prime with DNA or another AAV serotype (40, 41).

Here, we investigated the potential efficacy of AAV-vectored vaccines harboring either the *pfcsp*, *pfs25,* or fusion of both genes by applying a heterologous prime-boost vaccination regimen with other viral-vectored or protein-in-adjuvant vaccines. Transgenic *P. berghei* expressing either the *pfcsp* or *pfs25* genes was used to evaluate protective and TB efficacies in a murine model. Our results demonstrated that prime-boost delivery of *P. falciparum* pre-erythrocytic and sexual stage Ag by human adenovirus 5 (AdHu5) followed by adeno-associated virus serotype 1 (AAV1) is capable of inducing sustained high titer of Ab responses. In addition, I propose that, our vaccine regimen, especially the Ad-prime-

AAV-boost of the fusion Ag (multivalent vaccines) has the potential to fulfill the landmark goal of the malaria vaccine technology roadmap, by achieving sterile protection and long-term transmission blocking efficacies.

AIMS OF THE STUDY

The overall aim of this thesis was to examine the potential of adeno-associated virus serotype 1 (AAV1) as viral vector for malaria vaccines. In addition, the specific aims of the study were:

- To investigate whether AAV1 could drive the expression of malaria Ags in mammalian cells
- To study whether the AAV1 could induce Ag specific immune responses in mice model
- To identify the most immunogenic prime-boost regimen using AAV1
- To check the durability of immune responses conferred by AAV1 vaccination
- To evaluate the protective efficacy of vaccine regimen using AAV1 expressing PfCSP and multi-stage Pfs25-PfCSP
- To assess the transmission blocking efficacy of vaccine regimen using AAV1 expressing Pfs25 and multi-stage Pfs25-PfCSP

MATERIALS AND METHODS

Ethics statement

All animal care and handling procedures were approved by the Animal Care and Use Committee of Kanazawa University (No. AP-163700) and by the Guidelines for Animal Care and Use prepared by Jichi Medical University (No. 09193). All efforts were made to minimize suffering in the animals.

Parasites and animals

Transgenic *P. berghei* Pfs25DR3 used for TB assays was kindly donated by Andrew Blagborough from Imperial College London (42). Transgenic *P. berghei* expressing PfCSP (PfCSP-Tc/Pb) for the protective efficacy study was described previously (43, 44). Both transgenic parasites were maintained in the Laboratory of Vaccinology and Applied Immunology, Kanazawa University. *Anopheles stephensi* mosquitoes (SDA 500 strain) were infected with the transgenic parasites by allowing them to feed on parasite-infected 6-week-old ddY mice. All other animal experiments used 6-week-old BALB/c mice.

In vivo bioluminescent imaging

AAV1 expressing luciferase (AAV1-Luc) was administered into the right medial thigh muscles of BALB/c mice (n = 3; $1\times10_{11}$ viral genomes [vg]/mouse) on day 0, and D-Luciferin (15 mg/mL; OZ Biosciences, Marseille, France) was administered intraperitoneally (i.p.; 150 µL/mouse) at the appropriate timepoints. Luciferase expression was detected as described previously (44, 45). The accumulated emissions were calculated, and their intensities were expressed in a color heat map.

Viral vector construction

To generate AAV1-PfCSP-G(−) and AAV1-PfCSP-G(+), the gene cassette encoding the mouse IgGκ signal peptide, FLAG tag, and WPRE was first synthesized and cloned into pUC57-Simple, to construct pUC57-Simple-SP-FLAG-WPRE (GenScript, Piscataway, NJ, USA) (**Figure 1**). The codon-optimized gene encoding a GPI anchor-lacking PfCSP (Leu_{19}–Val_{377}) was excised from pENTR-CAG-sPfCSP2-G2-sWPRE by digestion with EcoRI and XmaI and then inserted into the MunI and XmaI sites of pUC57-Simple-SP-FLAG-WPRE to construct pUC57-sPfCSP2-WPRE. Next, the gene cassette encoding SP-FLAG-sPfCSP2-WPRE was excised from pUC57-sPfCSP2-WPRE, by digestion with EcoRI and XhoI and then inserted into the EcoRI and XhoI sites of pAAV-MCS under the control of

the CMV promoter sequence to construct pAAV-CMV-sPfCSP2-G(−). The gene encoding PfCSP with VSV-G was excised from pENTR-CAG-sPfCSP2-G2-sWPRE by digestion with BamHI and XhoI and then inserted into the BamHI and XhoI sites of pAAV-CMV-sPfCSP2-G(−) to construct pAAV-CMV-sPfCSP2-G(+). These plasmids, pAAV-CMV-sPfCSP2-G(−) and pAAV-CMV-sPfCSP2-G(+), were used to produce the AAV1-PfCSP-G(−) and AAV1-PfCSP-G(+), respectively, in HEK293 cells as described elsewhere (29).

To generate AdHu5-Pfs25, the codon-optimized genes encoding Pfs25 and G6S hinge were first synthesized and cloned into pUC57-Simple to construct pUC57-Simple-sPfs25-hinge (GenScript) (**Figure 1**). The *pfs25* gene fragment was then excised from pUC57-Simple-sPfs25-hinge by digestion with EcoRI and AgeI and inserted into the EcoRI and XmaI sites of pENTR-CAG-sPfCSP2-G2-sWPRE to construct pENTR-CAG-sPfs25-G2-sWPRE. This plasmid, pENTR-CAG-sPfs25-G2-sWPRE, was cloned into the shuttle vector pAd/PL-DEST (Invitrogen, Carlsbad, CA, USA) using the LR recombination reaction. The resulting adenovirus was purified and titrated using the Fast-Trap Adenovirus Purification and Concentration Kit (Millipore, Temecula, CA, USA) and the Adeno-X™ Rapid Titer Kit (Clontech, Palo Alto, CA, USA), respectively, according to the manufacturers' protocols. AdHu5-PfCSP has been described previously (46).

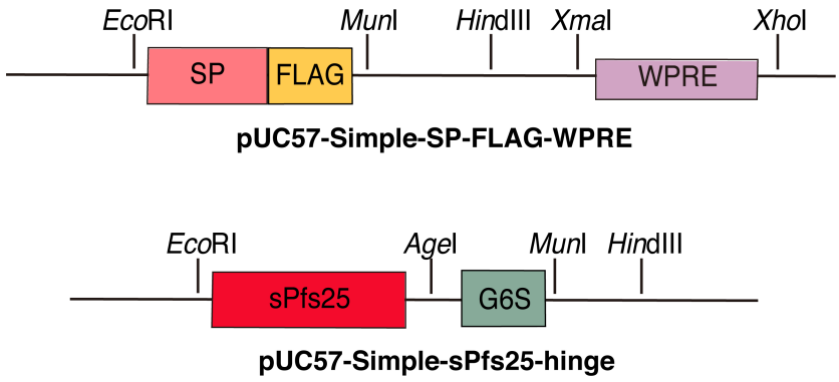

Figure 1: Construct of plasmids for generation of the viral-vectored vaccines.

To generate AAV1-Pfs25, the gene cassette encoding SP-FLAG-sPfs25-WPRE was first excised from pENTR-CAG-sPfs25-G2-sWPRE by digestion with EcoRI and XhoI and then inserted into the EcoRI and XhoI sites of pAAV-MCS under the control of the CMV promoter sequence, to construct pAAV-CMV-Pfs25. This plasmid, pAAV-CMV-Pfs25, was subsequently used to produce AAV1-Pfs25 in HEK293 cells as described elsewhere (29).

For generation of AdHu5-Pfs25-PfCSP, the gene encoding Pfs25 and Gly_6Ser hinge was excised from pUC57-Simple-sPfs25-hinge (**Figure 1**) by digestion with EcoRI/MefI and then inserted into the EcoRI site of pENTR-D-sPfCSP2-G2-sWPRE (47) to construct

pENTR-D-sPfs25-sPfCSP-WPRE; The gene cassette encoding the fusion Pfs25-PfCSP was excised by digestion of pENTR-D-sPfs25-sPfCSP-WPRE with EcoRI/XmaI and then inserted into EcoRI/XmaI sites of pENTR-CAG-sPfCSP2-G2-sWPRE (paper in submission) to construct pENTR-CAG-sPfs25-sPfCSP2-G2-sWPRE which was subsequently cloned into the shuttle vector pAd/PL-DEST (Invitrogen, Carlsbad, CA, USA) using LR recombination reaction. The adenovirus was purified and titrated as described previously (46). For generation of AAV1-Pfs25-PfCSP, the gene cassette encoding the fusion Pfs25-PfCSP was excised from pENTR-CAG-sPfs25-sPfCSP2-G2-sWPRE by digestion with KpnI and XhoI and then inserted into the KpnI and XhoI sites of pAAV-CMV-sPfs25 (paper in submission). The resulting plasmid, pAAV-CMV-sPfs25-sPfCSP2 was used to generate AAV1-Pfs25-PfCSP in HEK293 cells as described elsewhere (29).

Immunoblotting

HEK293T cells were transduced with the Ad vaccines at a multiplicity of infection (MOI) of 3 or 10 or with the AAV1 vaccines at an MOI of 10^5 or 10^6 at 24 h after being seeded into plates. Cell lysates were collected using Laemmli buffer at 48 h post-infection and subjected to immunoblotting. The cell lysates were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels under reducing conditions for PfCSP and under non-reducing conditions for Pfs25. Samples were transferred electrophoretically onto an Immobilon FL® PVDF membrane (Merck Millipore, Tokyo, Japan). The membranes were blocked for up to 1 h using 5% skim milk in PBS containing 0.1% Tween 20 (PBS-T), then incubated for 1 h at room temperature with the monoclonal Ab (mAb) anti-PfCSP 2A10 or mAb anti-Pfs25 4B7, diluted 1:10,000 in 5% skim milk. After washing with PBS-T, blots were probed with the secondary Ab, goat anti-mouse conjugated to IRDye 800 (Rockland Immunochemicals, Limerick, PA, USA), diluted 1:20,000 in 5% skim milk. The membrane was visualized using an Odyssey infrared imager (LI-COR, Lincoln, NE, USA). The molecular weight predictions were performed using the ExPASy server.

Immunofluorescence assay (IFA)

For IFA of protein expression, HEK293T cells were infected with the Ad vaccines or AAV1 vaccines on an 8-well chamber slide at an MOI of 10 or 10^5, respectively. Cells were fixed with 100% methanol or 4% paraformaldehyde for 30 min at 24 h post-infection. To visualize the expression of the Ag, the cells were incubated for 1 h at room temperature with Alexa Fluor 488-conjugated 2A10 or Alexa Fluor 596-conjugated 4B7, diluted 1:100. For IFA of the sporozoite and ookinete, the sporozoites isolated from mosquito salivary

glands were loaded onto glass slides and fixed with acetone/methanol (1:1) while the ookinete isolated from infected blood were fixed with 4% paraformaldehyde. Slides were blocked with 10% normal goat serum before incubated with sera from immunized mice (1:80 dilution) for 1 h, followed by incubation with FITC conjugated goat anti-mouse IgG for 1 h. VECTASHIELD® containing 4′, 6-diamidino-2-phenylindole was used for nuclei staining. For positive control, ookinetes and sporozoites were stained with Alexa Fluor 596-conjugated 4B7 and 2A10, respectively. A BZ-X710 fluorescence microscope (Keyence Corp, Tokyo, Japan) was used for image acquisition of all IFA experiments.

Immunization

All vaccines were administered intramuscularly in 100 µL of PBS. Ad vaccines were administered at a dose of 5×10^7 plaque-forming units (PFU), while AAV1 vaccines were administered at a dose of 10^{11} vg per mouse. Insect baculovirus expressing PfCSP (BV-PfCSP) was administered at 10^8 PFU and recombinant full-length PfCSP (rPfCSP) was administered at 10 µg in Imject® Alum (Thermo Scientific, Waltham, MA, USA). Immunization was done with 3 week- or 6 week-interval between prime and boost. For mixture regimen, AdHu5-PfCSP (5×10^7 PFU) and AdHu5-Pfs25 (5×10^7 PFU) were mixed in a syringe as a prime, while AAV1-PfCSP (10^{11} vg) and AAV1-Pfs25 (10^{11} vg) were mixed as a boost. The negative control was injected with either PBS or AdHu5 expressing luciferase (AdHu5-Luc)-prime/AAV1-Luc-boost (AdHu5-AAV1 Luc).

ELISA

PfCSP- or Pfs25-specific Ab levels were quantified by ELISA as previously described (44). The Pfs25 Ag, constructed using the same *pfs25* gene used in viral vectored vaccines, was produced using wheat germ cell-free (WGCF) protein expression system (CellFree Sciences, Matsuyama, Japan)(48), whereas the PfCSP Ag was produced using *E coli* expression system. Sera from immunized mice were collected from tail vein blood samples one day before boost and one day before the challenge, or weekly up to 280 days post-boost for monitoring. Pre-coated Costar® EIA/RIA polystyrene plates (Corning Inc, NY, USA) with 400 ng/well of PfCSP or 200 ng/well of Pfs25 were blocked with 1% bovine serum albumin (BSA) in PBS and then incubated with serially diluted sera samples, as well as with negative and positive controls (mAb 2A10 or mAb 4B7, respectively). An anti-mouse IgG conjugated with horseradish peroxidase (HRP) (Bio-Rad Lab, Inc, Tokyo, Japan) was used as a secondary Ab. Endpoint titers were expressed as the reciprocal of the last dilution

that gave an optical density at 414 nm of 0.15 U above the values of the negative controls (<0.1). All mice used in our experiments were seronegative before immunization.

Intracellular cytokine staining (ICS) and ex vivo interferon (IFN)-γ ELISPOT assay

ICS and ELISPOT were performed using splenocytes as described previously (46). For ICS, the splenocytes were stimulated with a final concentration of 1 μg/mL of the immunodominant $CD8^+$ T-cell epitope NYDNAGTNL ($PfCSP_{39–47}$) and 1 μg/mL of GolgiPlug™ (BD Biosciences, Tokyo, Japan) in a 96-well U-bottom tissue culture plate (Corning Inc.) for 6 h. The cells were then surface stained with anti-mouse CD16/32 Ab, Pacific Blue™-conjugated anti-mouse CD4 Ab, and PerCP/Cy5.5-conjugated anti-mouse CD8α Ab, and the cytokine was stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IFN-γ Ab or a FITC-conjugated rat IgG1κ isotype control Ab. Data were acquired with a BD FACSVerse™ Flow Cytometer (BD Biosciences) and analyzed with FlowJo (Tree Star, Ashland, OR, USA). All Abs were purchased from BioLegend (San Diego, CA, USA).

For ELISPOT, splenocytes were cultured for 20–24 h on an ELISPOT microplate (Perkin Elmer, Yokohama, Japan) with the H-$2K_d$-restricted PfCSP T-cell epitope (NYDNAGTNL, $PfCSP_{39–47}$; final concentration, 1 μg/mL) or the PfCSP-overlapping peptide pool (final concentration, 5 μg/mL). Results are expressed as IFN-γ spot-forming units (SFU) per million splenocytes.

Parasite challenge test

Mice were intravenously challenged with PfCSP-Tc/Pb sporozoites resuspended in RPMI-1640 media (Gibco, Life Technologies, Tokyo, Japan). Each mouse was injected with 100 μL of media containing 1,000 or 500 sporozoites via the tail vein. Infection was monitored from day 4 to 14 by Giemsa staining of thin blood smears obtained from the tail. Protection was defined as the complete absence of blood-stage parasitemia on day 14 post-challenge. Protective efficacy was calculated using the following formula: % protective efficacy = [1 − [(number of infected mice in the vaccine group/total number of mice in the vaccine group)/(number of infected mice in the non-immunized group/total number of mice in the non-immunized group)]] × 100. The time required to reach 1% parasitaemia was determined as described previously (49).

TB assays

TB was assessed using direct-feeding assays (DFAs). At 35 or 287 days after boost, mice were treated with phenylhydrazine (PHZ) and then infected i.p. with 10^6 *Pb*Pfs25DR3-

parasitized red blood cells (pRBCs) three days later. At 3 days post-infection, at least 50 starved *A. stephensi* mosquitoes were allowed to feed on each infected mouse. At 5–6 h post-feeding, any unfed mosquitoes were removed. Mosquitoes were then maintained on fructose [8% (w/v) fructose, 0.05% (w/v) *p*-aminobenzoic acid] at 19–22 °C and 50–80% relative humidity. On day 10–12 post-feeding, the mosquito midguts were dissected, and oocyst prevalence and intensity were recorded. For each mouse, the number of oocysts was counted, and the mean oocyst intensity was calculated. For inhibition calculations, these numbers were compared with those of mice immunized with AdHu5-AAV1 Luc control. Percent (%) inhibition of mean oocyst intensity (transmission-reducing activity; TRA) was calculated as follows: 100 × [1 − (mean number of oocysts in the test group/mean number of oocysts in the control groups)]. Similarly, the % inhibition of oocyst prevalence (transmission-blocking activity; TBA) was evaluated as 100 × [1 − (proportion of mosquitoes with any oocysts in the test group)/(proportion of mosquitoes with any oocysts in the control group)](50).

Statistical analysis

For all statistical analyses, GraphPad Prism version 7.0 for Mac OS was used. Depending on the data distribution, a Student's *t*-test, Mann-Whitney rank test, or Wilcoxon matched-pairs signed rank test was used for comparing two groups. For the analysis of differences among immunization groups, a Kruskal-Wallis test with Dunn's correction for multiple comparisons or Tukey's multiple comparison was used. All ELISA end-point titers were log10 transformed before analysis. The protection level was analyzed by a Fisher's exact test. The proportion of mice not reaching 1% parasitemia was analyzed using Kaplan-Meier log-rank (Mantel-Cox) test. The significance of TRA and TBA was assessed using the Mann-Whitney U test and Fisher's exact probability test, respectively. A *p*-value of <0.05 was considered statistically significant.

RESULTS

Long-lasting luciferase expression by AAV1-Luc at the injection site

It has been shown that AAV vectors are capable of expressing a transgene for a long time and of transducing skeletal muscle efficiently with minimal inflammatory response (29, 51). To examine the transduction efficacy and long-lasting transgene expression of our AAV1, AAV1-Luc was administered into the right medial thigh muscles of BALB/c mice (10^{11} vg/mouse; n = 3) on day 0. Luciferase expression was monitored with bioluminescence imaging (**Figure 2A**), and the data for the total flux (**Figure 2B**) at different timepoints were normalized against that at day 1. The luminescence signal increased gradually from day 0 to day 7, reaching the peak within ten days (10^{10} p/s/cm^2/sr); as expected, robust luciferase expression persisted for up to 260 days (**Figures 2A,B).** This result indicates that our AAV1 vector system can efficiently transduce muscle cells and achieve sustained expression of the transgene product in mouse muscle, consistent with other studies showing a high level and stable transgene expression after an i.m. injection of AAV serotype 1 or 2, lasting for 1 to 5 years (52-54).

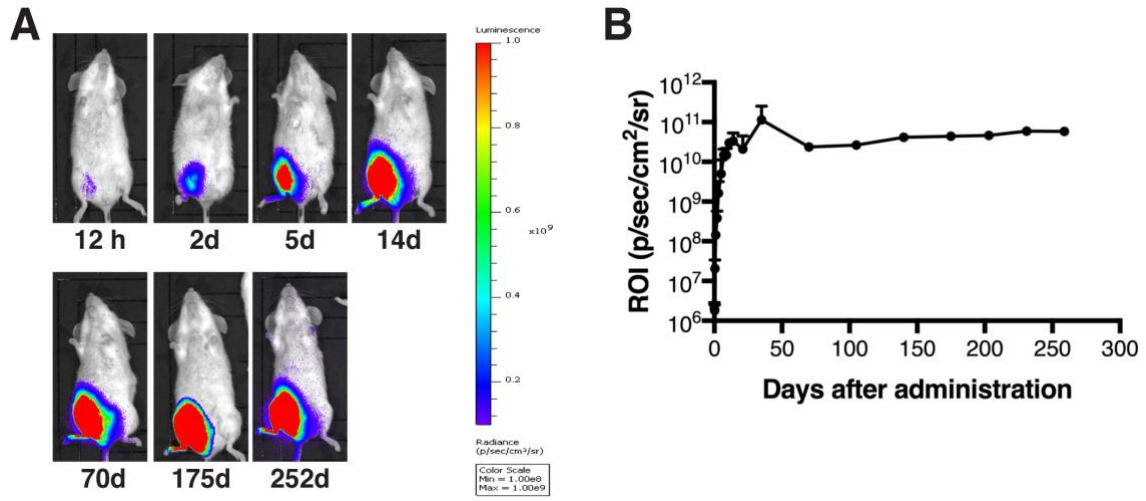

Figure 2: Long-term transgene expression after muscle transduction with AAV1-Luc. (A) Luciferase expression at different timepoints, detected by using the IVIS Lumina LT Series III *in vivo* imaging system. AAV1-Luc was administered into the right medial thigh muscles of BALB/c mice (n = 3; 1×10^{11} vg/mouse) on day 0. Luciferase expression remained high up to 252 days post-administration of AAV1-Luc. The heat map images visible in the mice represent the total flux of photons (p/s/cm^2) in that area. Rainbow scales are expressed in radiance (p/s/cm^2/sr). **(B)** The mean total flux of photons is shown as a region of interest (ROI) from day 0 to day 252 after administration of AAV1-Luc

Construction of AAV1-PfCSP-G(−) vaccine

For the construction of pre-erythrocytic vaccine (PEV), we generated AAV1-PfCSP-G(−) harboring the gene cassette encoding a GPI anchor-lacking PfCSP (Leu_{19}–Val_{377}), followed by a *wpre* sequence, under the control of the CMV immediate-early enhancer-promoter (**Figure 3A**). AAV1-PfCSP-G(−) was designed to allow PfCSP to be secreted from infected cells, which is a similar construction to that used in VIP. Immunoblotting revealed that the expression level of PfCSP in HEK293 cells increased gradually until 6 days after infection without any cytopathic effect (**Figures 3B, 4**); this is consistent with the expression pattern of luciferase shown in Figure 1. An IFA analysis showed that PfCSP was accumulated in the cytoplasm but not on the surface of infected cells (**Figure 3C**). Unexpectedly, no trace of PfCSP expression was detected in the cell medium (data not shown). These data indicate that PfCSP was not secreted from cells transduced with AAV1-PfCSP-G(−).

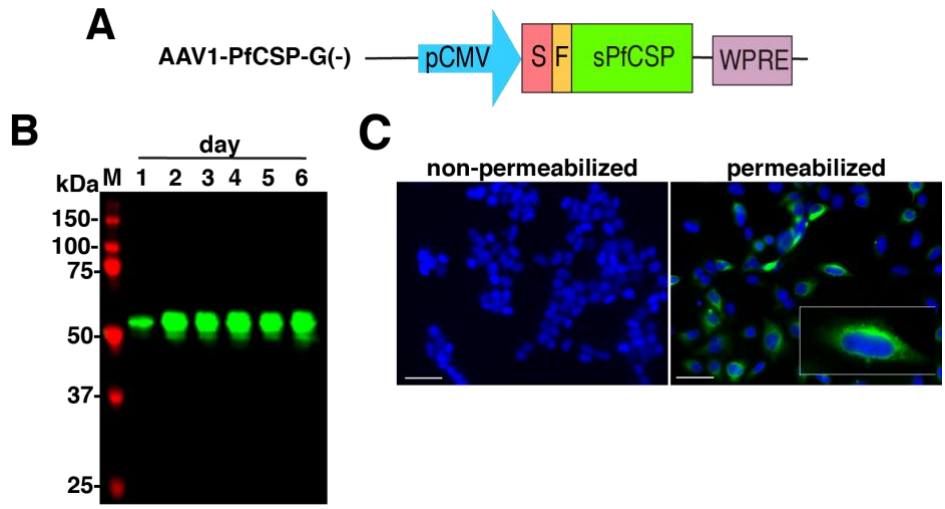

Figure 3: Functional activity of AAV1-PfCSP-G(−). (**A**) Construction of AAV1-PfCSP-G(−). Expression of the *pfcsp* gene cassette was driven by the CMV promoter. S, signal sequence; F, FLAG epitope tag. (**B**) Expression of PfCSP in HEK293T cells transduced with AAV1-PfCSP-G(−) (MOI = 10_5), as assessed by immunoblotting with anti-PfCSP mAb 2A10. M, molecular marker. (**C**) Localization of PfCSP expression in transduced cells. HEK293T cells were transduced with AAV1-PfCSP-G(−) (MOI = 10_5) as determined by IFA. After 48 h, cells were fixed with methanol (permeabilized) or paraformaldehyde (non-permeabilized) and incubated with Alexa-Fluor-488-conjugated mAb 2A10 (green). Cell nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI; blue). Original magnification, ×400. Bars = 50 µm.

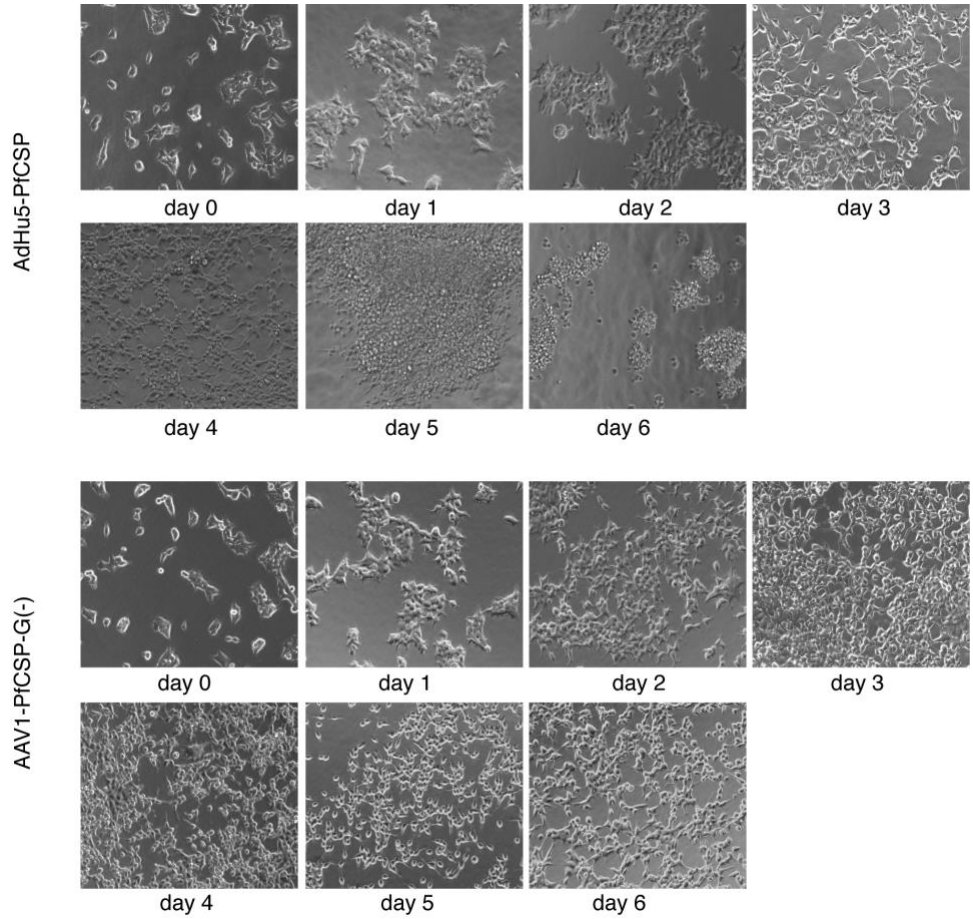

Figure 4: Cytopathic effect of AdHu5-PfCSP and AAV1-PfCSP-G(-). HEK293T cells were transduced with AdHu5-PfCSP (MOI = 1) or with AAV1-PfCSP-G(-) (MOI = 10_5). Interference pictures were taken up to 6 days post infection. Original magnification, ×200

Boosting with AAV1-PfCSP-G(−) following AdHu5-PfCSP priming induces potent and durable anti-PfCSP immune responses

We have previously generated rPfCSP in adjuvant vaccine capable of inducing protective immune responses as well as AdHu5-PfCSP and BV-PfCSP (25). To determine the optimal regimen of AAV1 immunization, we investigated several heterologous prime-boost immunization regimens using AAV1-PfCSP-G(−) in combination with these vaccines, in addition to the homologous AAV1, and we compared the induction of PfCSP-specific humoral and cellular immune responses. Mice were immunized with 3-week interval between prime and boost (n=3). At two weeks post-boost, their sera were collected for ELISA and their splenocytes were isolated for ICS and ELISPOT. The highest anti-PfCSP IgG titer was induced by the AdHu5-PfCSP-prime/AAV1-PfCSP-G(−)-boost heterologous regimen (AdHu5-AAV1 PfCSP) (**Figure 5A**). ICS assays revealed that AdHu5-AAV1 PfCSP induced

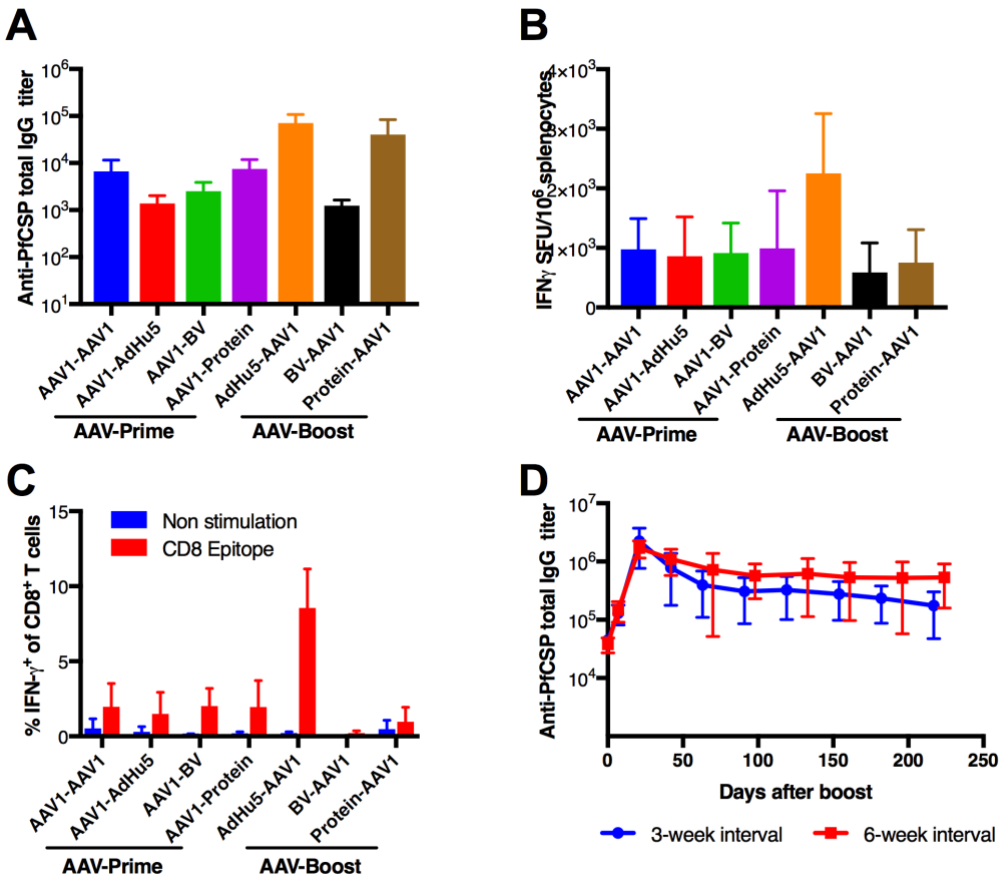

Figure 5: Kinetics of humoral and cellular immune responses. (**A**) Anti-PfCSP IgG antibody responses. BALB/c mice (*n* = 3) were immunized with the indicated regimens at a 3-week interval. Two weeks after boosting, serum samples were collected from each mouse, and their anti-PfCSP IgG titers were determined by ELISA. AdHu5-PfCSP, BV-PfCSP, AAV1-PfCSP-G(−), and rPfCSP protein are shown as AdHu5, BV, AAV1, and protein, respectively. Bars and error bars indicate the means and SD of the values, respectively (**B,C**) PfCSP-specific cellular immune responses. BALB/c mice were immunized as described in (A). At 2 weeks post-boost, splenocytes were stimulated with the synthetic PfCSP-specific $CD8_+$ T-cell epitope. (**B**) An ICS assay was performed on the splenocytes. Percentages of IFN-γ-secreting cells in the $CD8_+CD4_-$ T-cell population are shown after the subtraction of the percentages of cells stained with an isotype control antibody. (**C**) An e*x vivo* ELISpot assay was performed on splenocytes from the same mice. The IFN-γ SFU that reacted with the PfCSP-specific $CD8_+$ T-cell epitope per million splenocytes are shown. (**D**) Monitoring of anti-PfCSP IgG antibody responses. Groups of BALB/c mice (*n* = 5) were immunized with an AdHu5-PfCSP -prime and AAV1-PfCSP-G(−)-boost regimen at a 3-week or 6-week interval. Serum samples were collected from each mouse 1 day before boost and weekly after boost. Anti-PfCSP IgG titers were determined by ELISA and monitored for 224 days after booster injection.

the highest IFN-γ production by $CD8_+$ T cells after stimulation with the CSP-derived *H-2K*$_d$ peptide (**Figure 5B**). ELISPOT assays also showed that AdHu5-AAV1 PfCSP induced the highest frequency of IFN-γ-secreting cells, with an average of 2,252.22 SFU compared with <1,000 SFU per million splenocytes induced by other regimens (**Figure 5C**). Thus, although these experiments were not powered to detect statistically significant differences in immune

responses, the cellular immune responses demonstrated the same trend as seen in the humoral responses. Accordingly, we used the Ad-prime/AAV1-boost (AdHu5-AAV1) regimen for further vaccine evaluation. Ab monitoring showed that sustained high titer of anti-PfCSP IgG was induced for 224 days (**Figure 5D**).

To assess the protective efficacy of heterologous prime-boost regimens using AdHu5-PfCSP and AAV1-PfCSP-G(−), immunized mice were challenged with PfCSP-Tc/Pb sporozoites two weeks post-boost, and the presence of blood infection was evaluated up to 14 days post-challenge. For comparison, we also performed challenges with homologous AdHu5-PfCSP (Ad-Ad), homologous AAV1-PfCSP-G(−) (AAV-AAV), and heterologous prime-boost of AAV1-PfCSP-G(−)-prime/AdHu5-PfCSP-boost (AAV-Ad). The AdHu5-AAV1 immunization regimen conferred only a moderate sterile protection rate (37.5%), but this rate was the highest among the four tested regimens (**Table 1, experiment 1**).

Table 1. Protective efficacies of heterologous prime and boost regimens using AdHu5 and AAV1 vaccines against sporozoite challenge[a]

Prime	Boost	Protected/challenged (% protective efficacy[b])
(Exp. 1)		
PBS	PBS	2/10 (0)
AAV1-G(−)	AAV1-G(−)	2/10 (0)
AAV1-G(−)	AdHu5	2/10 (0)
AdHu5	AAV1-G(−)	5/10 (37.5)
AdHu5	AdHu5	3/10 (12.5)
(Exp. 2)		
PBS	PBS	0/10 (0)
AdHu5	AAV1-G(−)	2/10 (20)
AdHu5	AAV1-G(+)	8/10 (80)[c]

[a]AdHu5-PfCSP, AAV1-PfCSP-G(−), and AAV1-PfCSP-G(+) are shown as AdHu5, AAV1-G(−), and AAV1-G(+), respectively. Immunized mice were intravenously challenged with 1,000 (Exp. 1) or 500 (Exp. 2) PfCSP-Tc/Pb sporozoites and checked for blood-stage infections by microscopic examination of Giemsa-stained thin smears of tail blood. Protection was defined as the complete absence of blood-stage parasitemia on day 14 post-challenge.

[b]Protective efficacy was calculated as described in the *Materials and Methods.*

[c]Significant difference with the PBS group as determined using a Fisher's exact probability test ($p < 0.001$)

Construction of AAV1-PfCSP-G(+) vaccine

In an effort to improve the protective efficacy, we generated AAV1-PfCSP-G(+), which anchors PfCSP on the surface of infected cells via the VSV-G protein membrane anchor (**Figures 6A**). The glycoprotein G of VSV is a 70-kDa glycoprotein containing two asparagine-linked complex oligosaccharides and is positioned such that almost 90% of the polypeptide chain is external to the lipid bilayer, forming spikes on the surface of the virion (55, 56). Thus, fusing an Ag to VSV-G allows a more efficient display of the Ag on transduced cells (56). Transduction by AAV1-PfCSP-G(+) demonstrated the same expression pattern on the cell surface (**Figures 6C**) as that by AdHu5-PfCSP (46). Immunoblotting with a quantification analysis showed that the amount of PfCSP expressed following transduction by AAV1-PfCSP-G(+) at MOI = 10_5 (PfCSP-VSV-G: predicted *Mr* of 53.3 kDa;) was three times higher than that following transduction by AAV1-G(−) (PfCSP: predicted *Mr* of 43.9 kDa) (**Figure 6B,** lanes 3 and 2, respectively), which was similar to the amount of PfCSP induced by AdHu5-PfCSP (MOI = 3, lane 1).

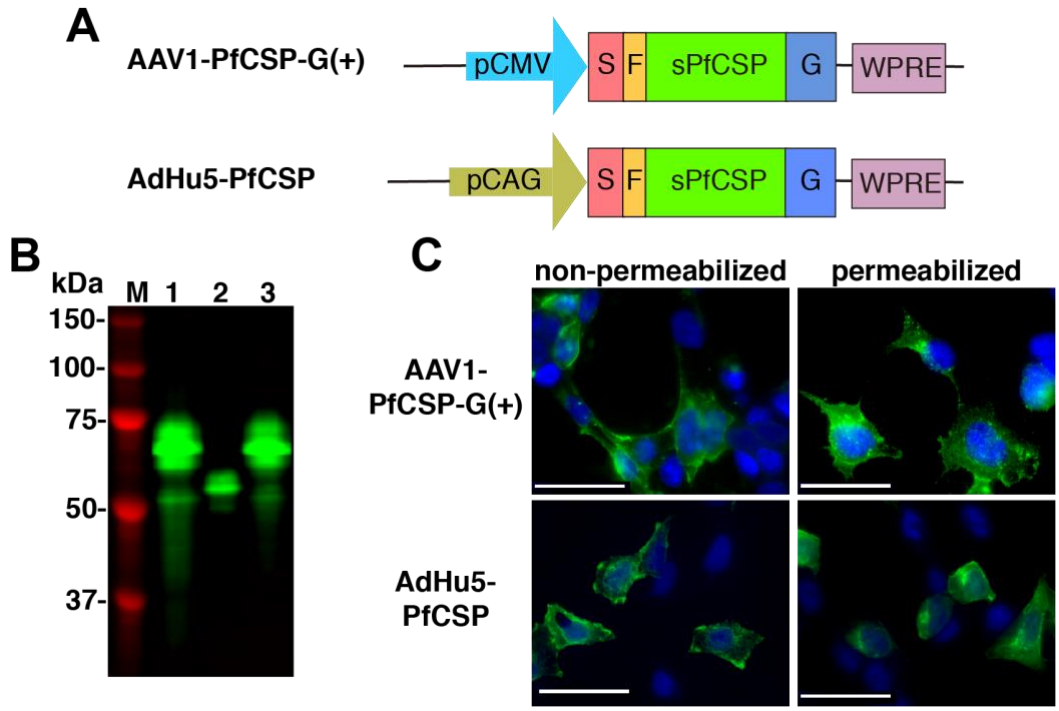

Figure 6: Functional activity of AAV1-PfCSP-G(+). (**A**) Constructs of AAV1-PfCSP-G(+) and AdHu5-PfCSP-G(+). Expression of the *pcsp* gene cassette in AAV1 and AdHu5 was driven by a CMV promoter and CAG promoter, respectively. G, VSV-G. (**B**) Expression of PfCSP in HEK293T cells transduced with AdHu5-PfCSP (lane 1, MOI = 3), AAV1-PfCSP-G(−) (lane 2, MOI = 10_5), or AAV1-PfCSP-G(+) (lane 3, MOI = 10_5), as assessed by immunoblotting with mAb 2A10 at 48 h post-transduction. (**C**) Localization of PfCSP expression in HEK293T cells transduced with AAV1-PfCSP-G(+) (MOI = 10_5) and AdHu5-PfCSP (MOI = 10), as determined by IFA conducted as described in Figure 3C.

Importantly, boosting with AAV1-G(+) following an AdHu5-prime evoked significantly higher anti-PfCSP IgG titers than did boosting with AAV1-G(−) ($1.15\times10_6$ vs $4.03\times10_6$, $p < 0.05$) (**Figure 7**). This result indicates that anchoring PfCSP through VSV-G enhanced not only the PfCSP expression level but also the humoral immune responses.

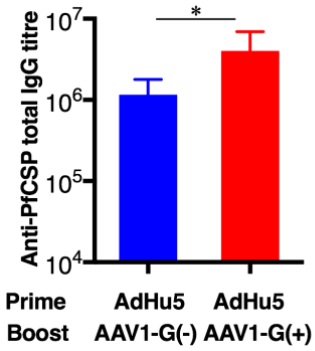

Figure 7: Anti-PfCSP IgG antibody responses. Groups of BALB/c mice (n = 10) were immunized with the indicated regimen at a 6-week interval. At 4 weeks post-boost, serum samples were collected from each mouse, and their anti-PfCSP IgG titers were determined by ELISA. AdHu5-PfCSP, AAV1-PfCSP-G(−), and AAV1-PfCSP (G+) are shown as AdHu5, AAV1-G(−), and AAV1-G(+), respectively. Bars and error bars indicate the means and SD of the values, respectively. Between-group differences were assessed with a Mann–Whitney U-test (*$p < 0.05$).

We then compared the boosting effects of AAV1-PfCSP-G(−) and AAV1-PfCSP-G(+) on protective efficacy. Mice immunized with a boost of either AAV1-PfCSP-G(−) or AAV1-PfCSP-G(+) following an AdHu5-PfCSP prime at 6-week interval were challenged with PfCSP-Tc/Pb sporozoites 5 weeks after boost. A significantly higher level of sterile immunity was achieved by AAV1-G(+) (80%) than by AAV1-G(−) (20%) (**Table 1, Experiment 2**). These results indicate that the display of PfCSP on the infected cells effectively enhanced the protective efficacy with the induction of a higher anti-PfCSP Ab response.

Construction and expression of AAV1-Pfs25 and AdHu5-Pfs25 vaccines

The ability of the AdHu5-AAV1 regimen to induce long-lasting high titer Abs led us to further explore this immunization regimen for the development of a TBV. With this aim, we generated AAV1-Pfs25 and AdHu5-Pfs25 expressing the pfs25 gene cassette fused to the VSV-G sequence **(Figure 8A**), which share similar construction with AAV1-PfCSP-G(+) and AdHu5-PfCSP, respectively. To examine the expression of conformationally dependent Pfs25 TB epitopes, Pfs25-VSV-G in HEK293T cells infected with AAV1-Pfs25 or AdHu5-

Pfs25 was analyzed by immunoblotting under non-reduced conditions using anti-Pfs25 mAb (4B7), which recognizes a conformation-dependent epitope of Pfs25 (57). 4B7 mAb reacted with Pfs25-VSV-G in cells infected with either virus as a ladder of bands with relative Mr of 33–48 kDa (**Figure 8B**, lanes 1 and 2). We hypothesize that these multiple bands may be due to post-translational modifications because there are two potential N-linked glycosylation sites in the predicted amino acid sequence of Pfs25-VSV-G. IFA analysis showed that Pfs25-VSV-G in cells infected with either virus was expressed not only in the cytoplasm but also on the surface of the cells (**Figure 8C**). These results suggest that the Pfs25-VSV-G on the surface of the infected cells might retain the three-dimensional structure of the native Pfs25 protein, which is essential for the induction of Abs with TB functionality (58).

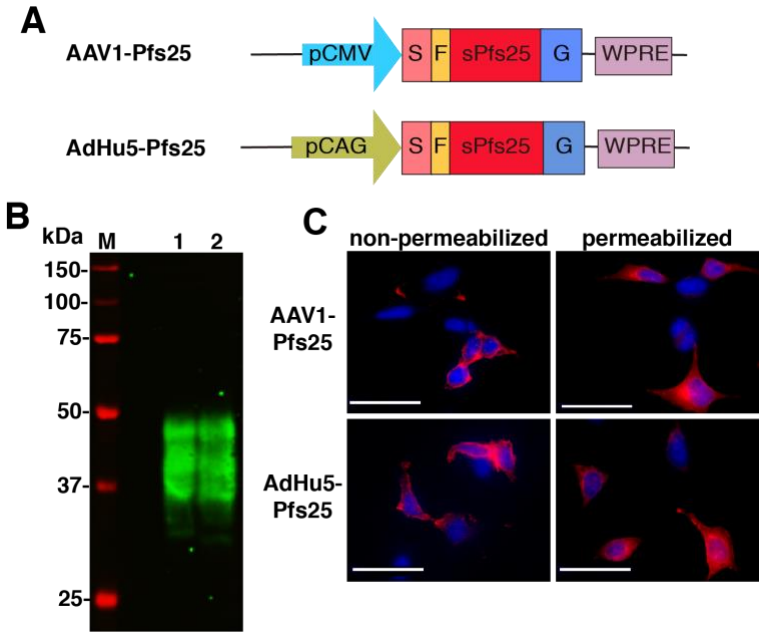

Figure 8: Functional activity of AdHu5-Pfs25 and AAV1-Pfs25. (**A**) Constructs of AdHu5-Pfs25 and AAV1-Pfs25. Expression of the *pfs25* gene cassette in AAV1 and AdHu5 was driven by a CMV promoter and CAG promoter, respectively. (**B**) Expression of Pfs25 in HEK293T cells transduced with AdHu5-Pfs25 (lane 1, MOI = 10) or AAV1-Pfs25 (lane 2, MOI = 10_6), as assessed by western blotting using anti-Pfs25 mAb 4B7. (**C**) Localization of Pfs25 expression in HEK293T cells transduced with AAV1-Pfs25 (MOI = 10_5) and AdHu5-Pfs25 (MOI = 10), as determined by IFA using Alexa-Fluor-594-conjugated 4B7 (red).

The AdHu5-AAV1 Pfs25 immunization regimen induces potent and durable anti-Pfs25 Ab responses

Since AdHu5-AAV1 PfCSP could induce long-lasting anti-PfCSP Ab responses for 280 days, we addressed whether AdHu5-AAV1 Pfs25 also possess this critical characteristic of a TBV. Anti-Pfs25 Ab responses in immunized mice were monitored for 280 days. Consistent with the response induced by AdHu5-AAV1 PfCSP, immunization with AdHu5-AAV1 Pfs25

similarly maintained high anti-Pfs25 IgG titers over 280 days (**Figure 9**)

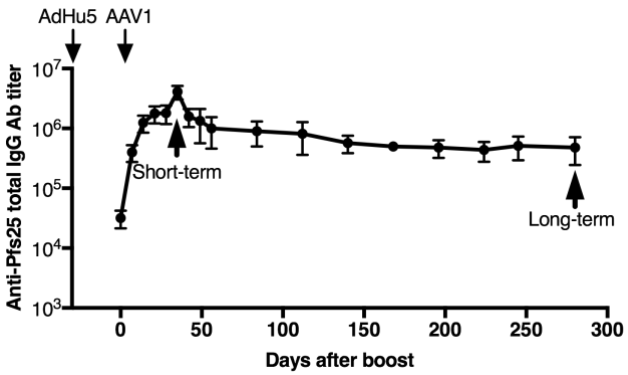

Figure 9: Monitoring of the anti-Pfs25 IgG titer. BALB/c mice (n = 5–10) were immunized with the indicated regimens at a 6-week-interval. Serum samples were collected from each mouse at 1 day before boost and weekly after boost. Anti-Pfs25 IgG titers were determined by ELISA and monitored for 280 days after booster injection. Right and left down arrows indicate prime and boost injections, respectively.

The AdHu5-AAV1 Pfs25 regimen elicits a long-lasting TB effect for 287 days

It has been widely accepted that the efficacy of transmission blockade relates directly to anti-Pfs25 Ab titers (59). To evaluate the functional activity of the anti-Pfs25 Ab induced by the immunization regimen, we assessed TB efficacies at 35 days (short-term) and 287 days (long-term) after boost by performing DFAs, which have been suggested to be about twice as effective at measuring TB as the standard membrane-feeding assay (SMFA) (60).

Groups of five mice were infected i.p. with 10_6 *Pb*Pfs25DR3-pRBCs. At three days after infection, three of the five mice were chosen for DFA by parasitemia (>2%) and gametocytemia (>0.05%) (**Figure 10**). *A. stephensi* mosquitoes were allowed to feed on each infected mouse, and the oocyst intensity and prevalence were recorded at 10–12 days post-feeding. Reduction in intensity and prevalence in the AdHu5-AAV1 Pfs25-immunized mice were calculated with respect to the AdHu5-AAV1 Luc-immunized controls.

A critical study suggested that TB assays should only be analyzed when controls have at least 35 oocysts per mosquito to obtain more reproducible data (61). In the short-term study, mosquitoes that fed on the three control mice displayed an average intensity of 125.17 oocysts/midgut. Following AdHu5-AAV1 Pfs25 immunization, the mean intensity was reduced to 0.20 oocysts/midgut, achieving a reduction (referred to as TRA) of 99.84% (p < 0.0001). Correspondingly, the mean infection prevalence was reduced from 97.18% to

10.41%, achieving a significant reduction (referred to as TBA) of 89.28% ($p < 0.0001$) (**Table 2, Figure 10A**).

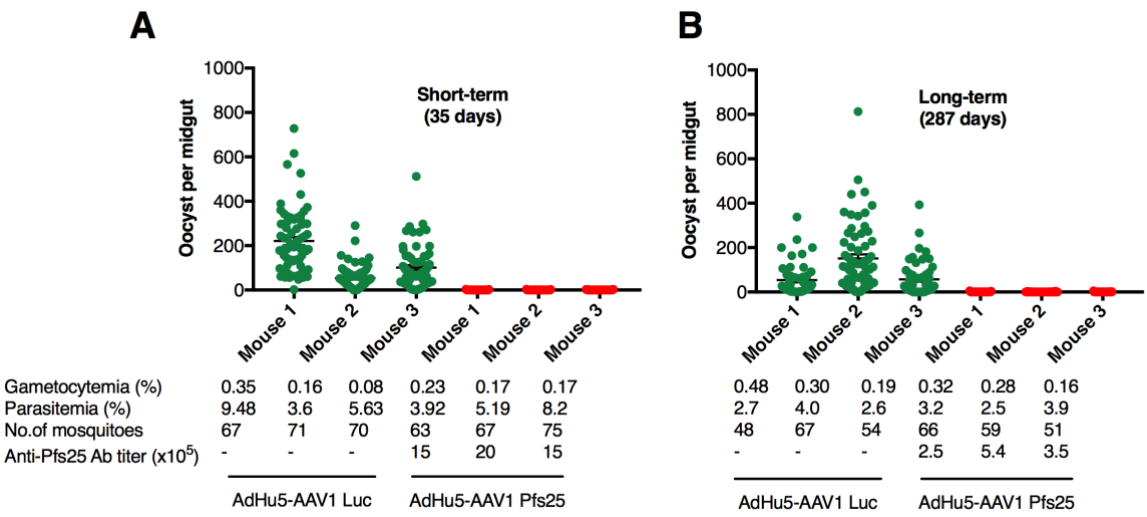

Figure 10: Transmission-blocking efficacy of the AdHu5-Pf25-prime and AAV1-Pfs25-boost regimen. (**A–B**) Groups of BALB/c mice ($n = 3$) were immunized with the indicated regimen at a 6-week interval and infected with Pfs25DR3 at 35 days (**A**) or 287 days (**B**) after boost. Mosquitoes were allowed to feed on the infected mice for a DFA. At day 10–12 post-feeding, the mosquito midguts were dissected, and oocyst intensity and prevalence were determined (**Table 2**). Each datapoint represents a single mosquito blood-fed on each mouse. The x-axis points represent individual mice. Horizontal lines indicate the mean numbers of oocysts observed (± standard errors of the means [SEM]).

Table 2. Transmission-blocking activity of AdHu5-Pfs25-prime/AAV1-Pfs25-boost immunization regimen

Group	Mean intensity ± SEM (oocysts per midgut)	Mean prevalence ± SEM (% infected mosquitoes)	TRA (%)[a]	TBA (%)[b]
Short-term (35 days)				
AdHu5-AAV1 Pfs25	0.20 ± 0.04	10.41 ± 3.51	99.84*	89.28*
AdHu5-AAV1 Luc	125.17 ± 49.84	97.18 ± 2.15		
Long-term (287 days)				
AdHu5-AAV1 Pfs25	0.24 ± 0.03	12.72 ± 1.53	99.73*	85.97*
AdHu5-AAV1 Luc	87.24 ± 31.86	90.63 ± 5.67		

[a]Transmission-reducing activity (TRA) was calculated by comparison with the control group, and significant differences were assessed using a Mann-Whitney U test (*$p <$ x0.0001)

[b]Transmission-blocking activity (TBA) was calculated by comparison with the control group, and significant differences were assessed using a Fisher's exact probability test (*$p < 0.0001$)

In the long-term study, although the mean IgG titer was reduced to about 20% compared with that at day 35, long-term TRA did not significantly decline over 287 days. Mosquitoes that fed on the three control mice displayed an average intensity of 87.24 oocysts/midgut, whereas the mean intensity following AdHu5-AAV1 Pfs25 immunization was reduced to 0.24 oocysts/midgut, achieving a TRA of 99.72% ($p < 0.0001$). Correspondingly, the mean infection prevalence was reduced from 90.63% to 12.72%, achieving a significant TBA of 85.97% ($p < 0.0001$) (**Table 2, Figure 10B**)

Both AdHu5-Pfs25-PfCSP and AAV1-Pfs25-PfCSP vaccines expressed Pfs25-PfCSP fusion Ag on the surface of mammalian cells in vitro

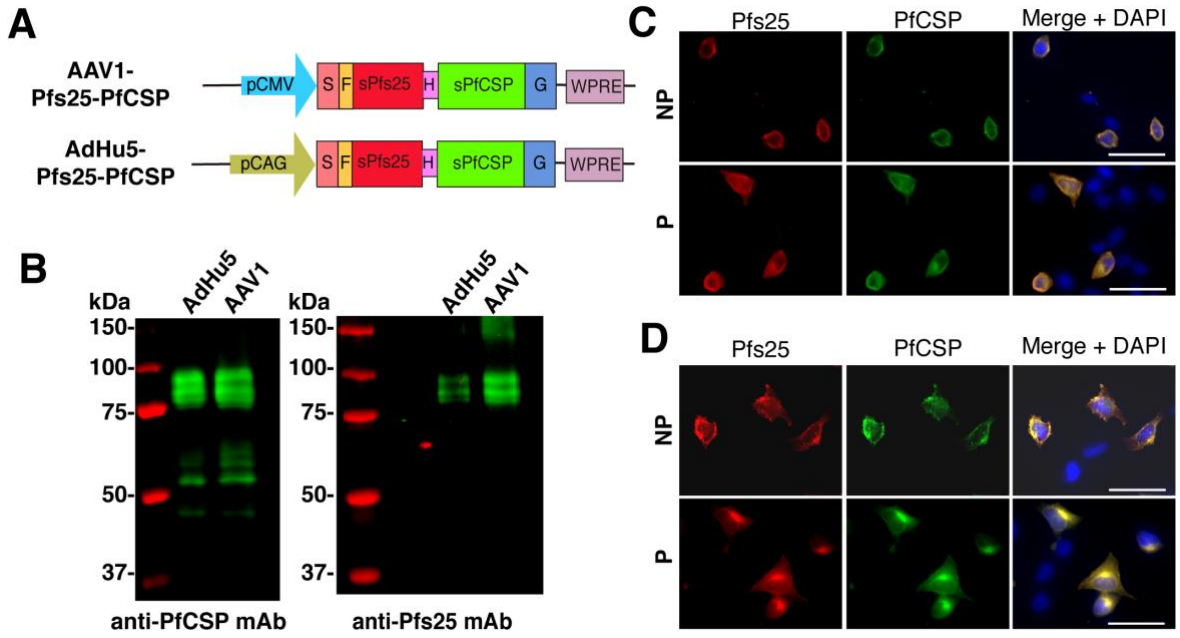

Figure 11: Construction of viral-vectored vaccines. (**A**) Expression of the *pfcsp* and *pfs25* gene cassette was driven by CMV promoter in AAV1, and by CAG promoter in AdHu5. S, signal sequence; F, FLAG epitope tag; H, Hinge G6S, G, VSV-G transmembrane protein. (**B**) Analysis of expression of PfCSP and Pfs25 in HEK293T cells transduced with AdHu5-Pfs25-PfCSP (MOI = 10) or AAV1-Pfs25-PfCSP (MOI = 10_6). Cells were lysed and loaded onto a 10% SDS-PAGE and immunoblotted with anti-PfCSP mAb 2A10 and anti-Pfs25 mAb 4B7. (**C,D**) Localization of PfCSP and Pfs25 expression in mammalian cells after transduction with AdHu5-Pfs25-PfCSP (**C**) or with AAV1-Pfs25-PfCSP (**D**). After 24 h, the cells were fixed with paraformaldehyde (NP; non-permeabilized) or methanol (P; permeabilized) and blocked with 10% NGS. After blocking, cells were incubated with Alexa-Fluor-488-conjugated anti- PfCSP mAb (green) and Alexa-Fluor-594-conjugated anti-Pfs25 mAb (red). Cell nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI; blue). Original magnification, ×400. Bars = 50 μm.

Expression of the *pfs25-pfcsp* fusion gene was driven by CAG and CMV promoter in AdHu5 and AAV1, respectively (**Figure 11A**). Immunoblotting revealed that the expression level of the Pfs25-PfCSP fusion Ag in the cells infected with AdHu5 (MOI = 10,

lane 1) is equal with that of infected with AAV1 (MOI = 10_6, lane 2) with relative Mr of 71.5 kDa (**Figure 11B**). The mAb 4B7 recognizes a conformation-dependent epitope of Pfs25, demonstrated that thePfs25-PfCSP fusion Ag maintain the correctly folded Pfs25 protein. IFA analysis showed that both Pfs25 and PfCSP epitopes were expressed in the cytoplasm and also on the surface of infected cells (**Figure 11C,D**). These results suggest that, similar with the Pfs25-VSV-G in the AdHu5-Pfs25 and AAV1-Pfs25, the Pfs25-PfCSP fusion Ag on the surface of the infected cells might retain the three-dimensional structure of the native Pfs25 protein, which is essential for the induction of Abs with TB functionality (58).

The AdHu5 prime-AAV1 boost induces potent and durable anti-PfCSP and anti-Pfs25 immune responses

To investigate the immunogenicity of the immunization regimen, first we determined the PfCSP- and Pfs25-specific Ab responses induced by the fusion vaccines compared with the mixture of single-Ag vaccines. Mice were immunized with a 6-week interval between prime and boost (*n*=15-28 for PfCSP analysis; *n*=5-8 for Pfs25 analysis). At four weeks post-boost, their sera were collected for ELISA. Both anti-PfCSP and anti-Pfs25 IgG titer were induced by the AdHu5-Pfs25-PfCSP-prime/AAV1-Pfs25-PfCSP-boost heterologous regimen (AdHu5-AAV1 Pfs25-PfCSP), comparable with those induced by the mixture of single-Ag vaccines (**Figure 12A**). Ab monitoring showed that the titers did not considerably reduce after 280 days (**Figure 12B**). IFA result demonstrated that immune sera from mice vaccinated with the AdHu5-AAV1 Pfs25-PfCSP reacted with the transgenic sporozoites (**Figure 12C**) and ookinetes (**Figure 12D**), as strong as that from mice immunized with the mixture of single-Ag vaccines.

The AdHu5-AAV1 Pfs25-PfCSP confers complete protection against transgenic *P berghei* expressing PfCSP

To assess the protective efficacy of the AdHu5-AAV1 Pfs25-PfCSP, immunized mice were challenged with PfCSP-Tc/Pb sporozoites four weeks post-boost, and the presence of blood infection was evaluated up to 14 days post-challenge. For comparison, we also performed challenges to the mice immunized with the mixture regimen. The AdHu5- AAV1 Pfs25-PfCSP conferred 57% protection, comparable with that of the mixture regimen (**Figure 13**). The protection level reached 100% in another challenge experiment.

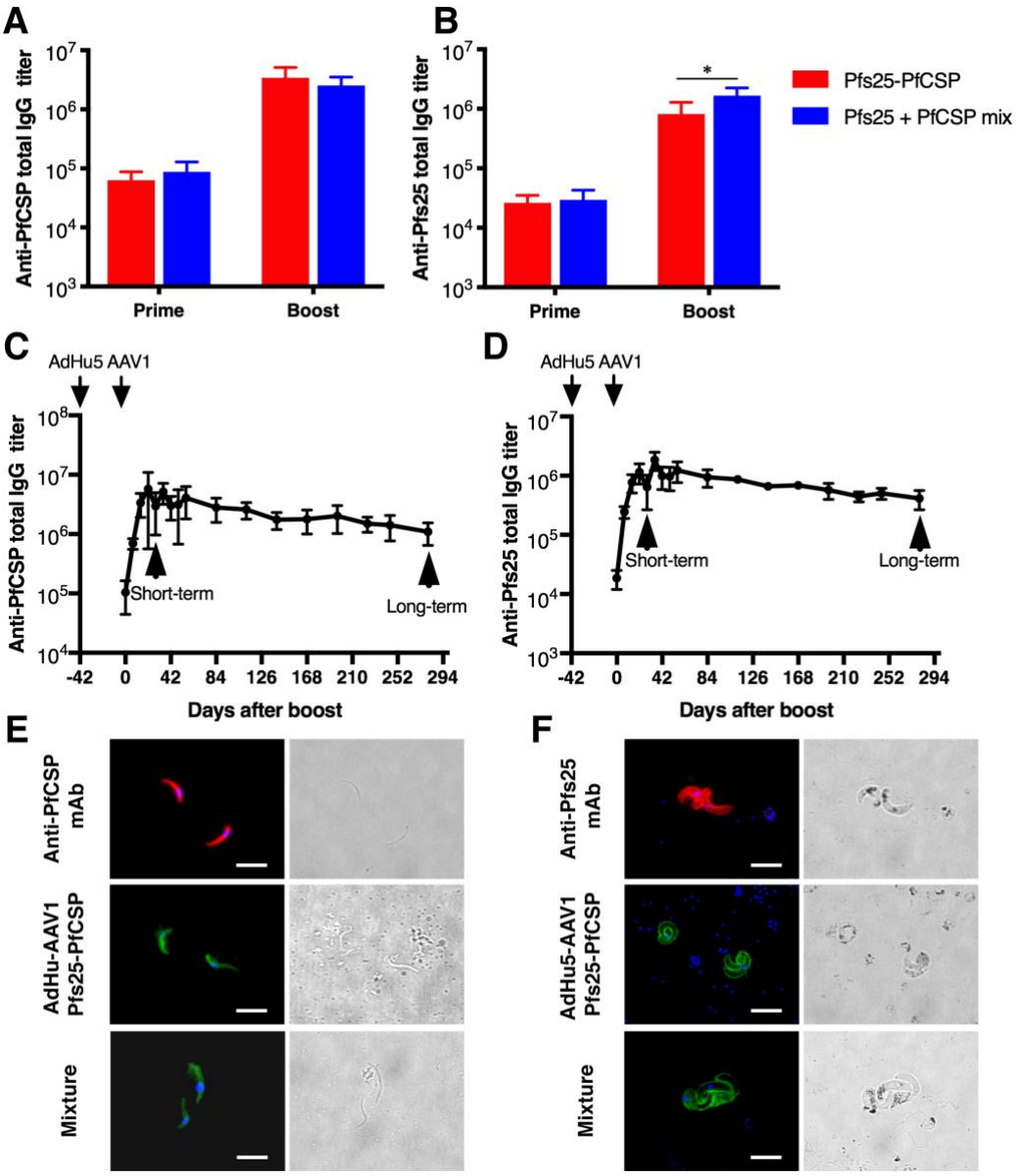

Figure 12: Immunogenicity of AdHu5-AAV1 Pfs25-PfCSP. (A,B) Comparison of antibody responses induced by the multi-antigen vaccine and the mixture of single antigen vaccine. BALB/c mice were immunized with the indicated regimen. Individual sera were collected 4 weeks after boost and antibody titers against PfCSP (A) and Pfs25 (B) were measured using ELISA. Data are represented as mean ± SEM. AdHu5-Pfs25-PfCSP-prime and AAV1-Pfs25-PfCSP-boost are shown as Pfs25-PfCSP; the mixture of AdHu5-Pfs25 and AdHu5-PfCSP prime and a mixture of AAV1-Pfs25 and AAV1-PfCSP boost are shown as Pfs25+PfCSP mix. Bars and error bars indicate the means and SD of the values, respectively. Differences between groups were assessed with Mann-Whitney U test; *p<0.05. (**C,D**) Monitoring of antibody responses. BALB/c mice (n = 5-10) were immunized with the AdHu5-AAV1 Pfs25-PfCSP at 6-week interval**.** Individual sera were collected a day before boost and weekly after the boost up to 280 days. Antibody titers against PfCSP (C) and Pfs25 (D) were measured using ELISA. Data are represented as mean ± SD. (**E,F**) Reactivity of immune sera with the transgenic parasites. The transgenic PfCSP-Tc/Pb sporozoite (**E**) and ookinete of Pfs25DR3 P*b* (**F**) were fixed and incubated with sera from immunized mice in (A,B) and stained with FITC conjugated goat anti-mouse IgG (green) for IFA. The sporozoites and ookinetes were incubated with Alexa-594-conjugated 2A10 and 4B7 for positive controls, respectively. Cell nuclei were visualized with 4,6-diamidino-2-phenylindole (DAPI; blue). Original magnification, ×1000. Bars = 10 μm.

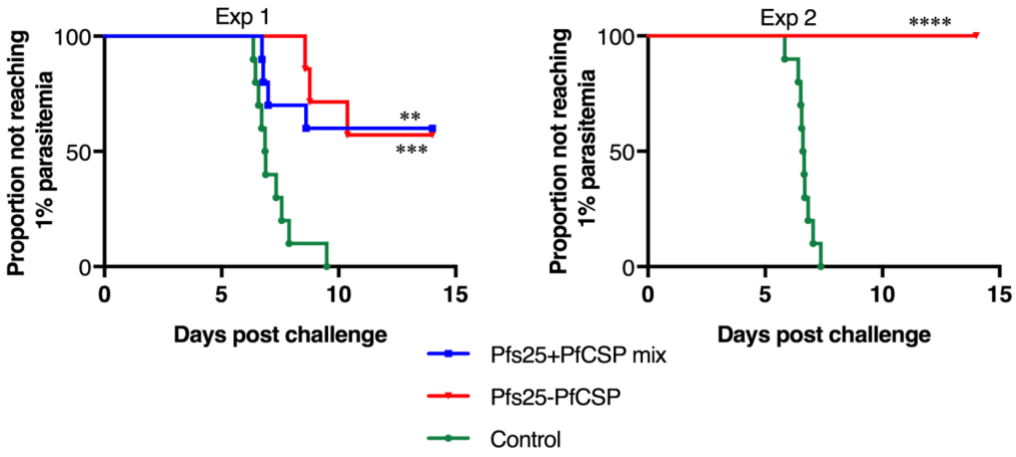

Figure 13: Protective efficacy after immunization with AdHu5-AAV1 Pfs25-PfCSP. BALB/c mice (n = 10) were immunized at 6-week-interval and challenged with an intravenous injection of 500 transgenic PfCSP-Tc/Pb sporozoites 4 weeks after boost. Parasitaemia was monitored for 3 consecutive days, starting from day 4 after challenge, and a model predicting the time to reach 1% parasitaemia was generated. The absence of blood-stage parasites in the animals was confirmed on day 14 after challenge. The statistical analysis was performed with Kaplan-Meier survival curves, and p values were calculated with a Kaplan-Meier log-rank (Mantel-Cox) test. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$ compared with control groups

The AdHu5-AAV1 Pfs25-PfCSP regimen elicits a long-lasting TB efficacy for 287 days

It has been widely accepted that the efficacy of transmission blockade relates directly to anti-Pfs25 Ab titers (59), thus we expected a high TB efficacies of the regimen. To evaluate the functional activity of the anti-Pfs25 Ab, we assessed TB efficacies at 35 days (short-term) and 287 days (long-term) after boost by performing DFAs. Groups of five mice were infected i.p. with 10_6 *Pb*Pfs25DR3-pRBCs. At three days after infection, three of the five mice were chosen for DFA by parasitaemia (>2%) and gametocytemia (> 0.05%) (**Figure 14**). *A. stephensi* mosquitoes were allowed to feed on each infected mouse, and the oocyst intensity and prevalence were recorded at 10–12 days post-feeding. Reduction in intensity and prevalence in the AdHu5-AAV1 Pfs25-PfCSP immunized mice were calculated with respect to the AdHu5-AAV1 Luc-immunized controls.

First, we evaluated the short-term TB efficacy of the dual Ag vaccines compared with the mixture of single-Ag vaccines (**Table 3 Exp.1, Figure 14A**). In this experiment, mosquitoes that fed on the three control mice displayed an average intensity of 61.87 oocysts/midgut. Following AdHu5-AAV1 Pfs25-PfCSP immunization, the mean intensity was reduced to 0.29 oocysts/midgut, achieving a reduction (referred to as TRA) of 99.53% ($p < 0.0001$), compared with 0.16 oocysts/midgut in the mixture group (TRA of 99.74%, $p < 0.0001$). Correspondingly, the mean infection prevalence was reduced from 83.59% to 6.94%

and 5.91% in the AdHu5-AAV1 Pfs25-PfCSP group and the mixture group, respectively, achieving a significant reduction (referred to as TBA) of 91.70% ($p < 0.0001$) and 92.93% ($p < 0.0001$). These results demonstrated that there are no significant differences in the TRA and TBA between the dual Ag group and the mixture group ($p = 0.67$ and $p = 0.82$, respectively).

Next, to re-confirm the TB efficacy of the AdHu5-AAV1 Pfs25-PfCSP, we performed another short-term study (**Table 3 Exp.2, Figure 14B**). In this experiment, mosquitoes that fed on the three control mice displayed an average intensity of 125.17 oocysts/midgut and infection prevalence of 97.18%. The mean intensity was 0.29 oocysts/midgut in the immunized group, gaining a TRA of 99.77% ($p < 0.0001$), and the mean infection prevalence was 8.04%, gaining a TBA of 91.73% ($p < 0.0001$). These result are comparable with those of exp 1.

Finally, we evaluated the long-term TB efficacy of the AdHu5-AAV1 Pfs25-PfCSP after 287 days of the booster injection (**Table 3 Exp3, Figure 14C**). The experiment revealed that TRA did not significantly decline over 287 days. The mean oocyst intensity in the immunized group was 0.42 oocysts/midgut compared to 87.24 oocysts/midgut of the control group, reaching a TRA of 99.52% ($p < 0.0001$), whereas the mean infection prevalence was 16.44 % compared to 90.63% of the control group, reaching a TBA of 81.87% ($p < 0.0001$).

Table 3. Transmission-blocking activity of AdHu5-Pfs25-PfCSP prime/AAV1-Pfs25-PfCSP boost immunization regimen

Group	Mean intensity ± SEM (oocysts per midgut)	Mean prevalence ± SEM (% infected mosquitoes)	TRA (%)[a,b]	TBA (%)[c,d]
Exp.1.Short-term (35 days)				
AdHu5-AAV1 Pfs25-PfCSP	0.29(0.07)	6.94 (2.47)	99.53*	91.70*
AdHu5-AAV1 Pfs25 + Ad- AAV PfCSP mix	0.16 (0.05)	5.91 (1.98)	99.74*	92.93*
AdHu5-AAV1 Luc	61.87 (26.92)	83.59 (11.21)		
Exp.2. Short-term (35 days)				
AdHu5-AAV1 Pfs25-PfCSP	0.291 (0.04)	8.037 (1.00)	99.77*	91.73*
AdHu5-AAV1 Luc	125.2 (49.84)	97.18 (2.15)		

Exp.3. Long-term (287 days)				
AdHu5-AAV1 Pfs25-PfCSP	0.42 (0.19)	16.44 (4.90)	99.52*	81.87*
AdHu5-AAV1 Luc	87.24 (31.86)	90.63 (5.67)		

a Transmission reducing activity (TRA) was calculated by comparison with the control (AdHu5-AAV1 Luc) group, and significant differences were assessed using Mann-Whitney U test (*$p < 0.0001$)

b No significant difference between TRA of Pfs25-PfCSP and Pfs25+PfCSP mix ($p = 0.6720$) in exp 1

cTransmission blocking activity (TBA) was calculated by comparison with the control group, and significant differences were assessed using a Fisher's exact probability test (*$p < 0.0001$)

d No significant difference between TBA of Pfs25-PfCSP and Pfs25+PfCSP mix ($p = 0.8171$) in exp 1

Collectively, our data demonstrate that AAV1 is an excellent booster vaccine vector following an AdHu5 prime to induce a high level of humoral immune responses and to achieve a high level of protective immunity and TB immunity against the malaria parasite. In addition, AdHu5-AAV1 Pfs25-PfCSP is an effective multi-stage malaria vaccines to induce a high level of PfCSP- and Pfs25- specific Ab immune responses and to achieve a high level of protective immunity and long-term TB immunity against the malarial parasites.

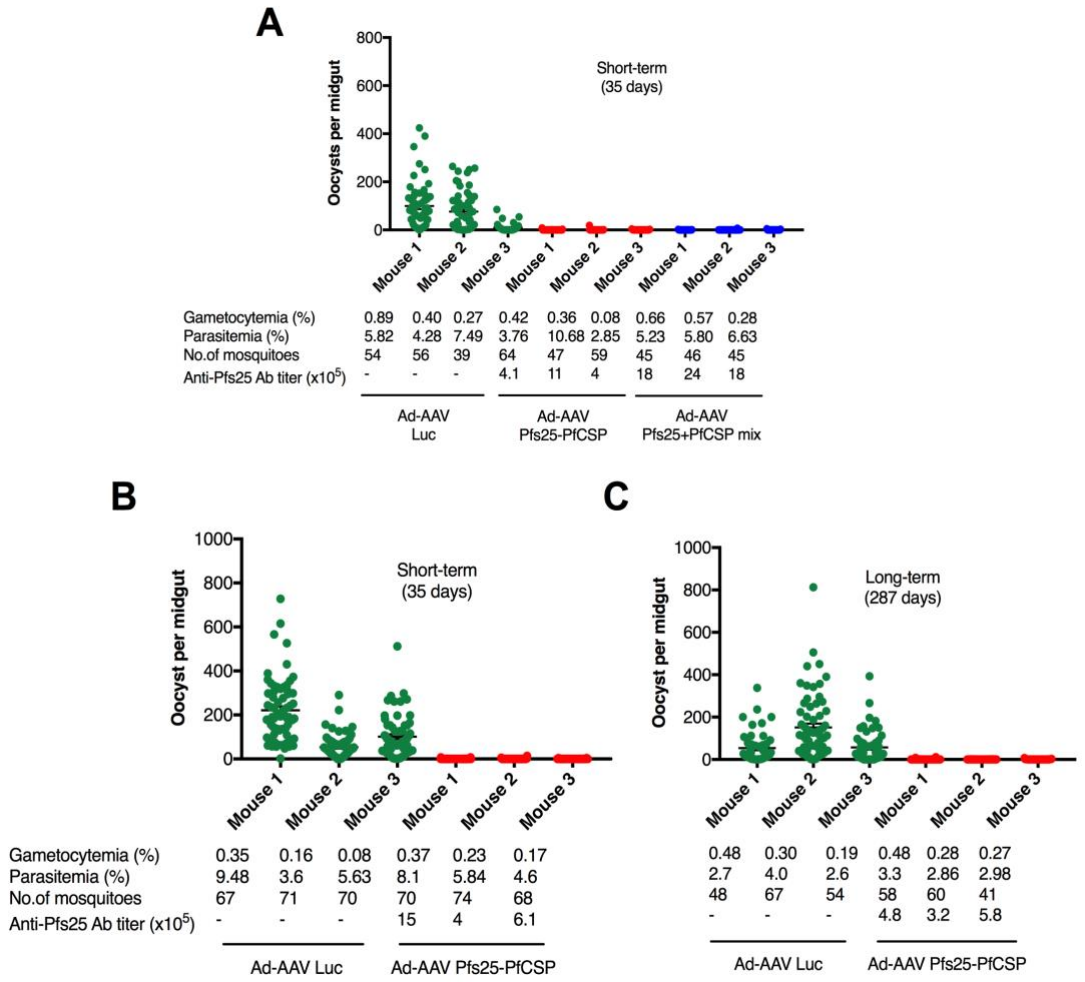

Figure 14: Transmission-blocking efficacy against Pfs25DR3 by AdHu5-AAV1 Pfs25-PfCSP. BALB/c mice were immunized with the indicated regimen at 6 week-interval (n = 3) and infected with Pfs25DR3 Pb 35 days (**A,B**) and 287 days (**C**) after boost. AdHu5-Pfs25-PfCSP-prime/AAV1-Pfs25-PfCSP-boost is shown as Pfs25-PfCSP; AdHu5-Pfs25 mixture with AdHu5-PfCSP prime/AAV1-Pfs25 mixture with AAV1-PfCSP boost is shown as Pfs25+PfCSP mix. Mosquitoes were allowed to feed on the infected mice by a direct-feeding assay. At day 10-12 post-feeding, mosquito midguts were dissected, and oocyst intensity and prevalence were determined [Table 1, exp 1(**A**), exp 2 (**B**), and exp 3(**C**)]. Each data point represents a single mosquito blood-fed on each mouse. X-axis points represent individual mice. Horizontal lines indicate the mean numbers of oocysts observed (± standard errors of the means [SEM]).

DISCUSSION

In this study, we demonstrated durable PfCSP- and Pfs25-specific humoral responses elicited by a heterologous AdHu5-prime and AAV1-boost immunization regimen. We evaluated the efficacies of these immunizations by using transgenic *P. berghei* parasites expressing either PfCSP or Pfs25 in a murine model, which facilitates the optimization of vaccine immunogenicity *in vivo* (62). The regimen targeting pre-erythrocytic stage Ag PfCSP elicited a high level of complete protection against sporozoite challenge. In the same way, the regimen targeting the mosquito-stage Ag Pfs25 conferred excellent TB activity as assessed using DFAs. Most notably, the TB activity was sustained up to 287 days after booster injection, with a TRA of 99%, fulfilling the requirement for an ideal TBV. Thus, AAV-based booster vaccines possess remarkable characteristics of inducing long-lasting Ab responses to major malaria vaccine candidate Ags following their administration after an AdHu5-priming vaccine.

It has been suggested that anti-CSP Ab titers are surrogate markers of protection for the magnitude and duration of RTS,S/AS01 efficacy (63). Waning anti-CSP Ab titers predict the duration of efficacy against clinical malaria. RTS,S/AS01 has shown reduced efficacy from 36.3% to 4.45% over a seven-year follow-up (2, 3). Therefore, to achieve more durable protective efficacy, it is necessary to develop vaccines capable of inducing sustained anti-CSP Abs. In the present study, we showed that using an AAV1-boost after an Ad-prime evoked a long-term high titer of anti-PfCSP. In addition, the regimen consists of only two doses, rather than the three doses needed for RTS,S; this reduced requirement will improve overall adherence to the vaccination schedule.

Regarding the use of this modality as a TBV, we demonstrated that the two-dose regimen of AdHu5-AAV1 Pfs25 elicited durable anti-Pfs25 Ab with a high level of TB efficacy over 287 days after booster injection. Pfs25, a 25-kDa surface Ag of zygotes and ookinetes, is currently the most developed TBV candidate that has been tested in human clinical trials (64). However, using this Ag for the development of TBVs is challenging, as the Ab titer cannot be boosted by natural infection (13, 14). Furthermore, a high concentration of anti-Pfs25 IgG is required to achieve significant blocking (64). Cheru *et al.* found that the Ab concentration needed to reduce the number of oocysts by 50% in a SMFA was 85.6 μg/mL (65). Conjugation of Pfs25 to *Pseudomonas aeruginosa* exoprotein A (EPA) with Alhydrogel (66) improved the immunogenicity of the vaccine and induced a geometric

mean of 88 μg/mL of anti-Pfs25 Ab in the highest dose group at two weeks after the fourth vaccination in a Phase I trial (67). However, the Ab levels declined to near baseline within 1 year of vaccination. As the TBA of anti-Pfs25 Abs correlates with the Ab titer, it is necessary for a TBV candidate to induce a sustained high titer of anti-Pfs25 IgG. In the present study, our AdHu5-AAV1 Pfs25 immunization regimen achieved this highly desirable attribute.

An earlier AAV-based malaria vaccine development failed to achieve a sufficient Ab titer for malaria protection, even when the AAV was used for boosting after a prime with another AAV serotype or with a DNA (40). Our strategy to combine the vaccine with an Ad-based vaccine in a heterologous prime-boost regimen revealed that AAV has the potential to induce Ab against the encoded Ag, particularly when administered as a boost, but not when administered as the prime. Although both AdHu5 and AAV1 have been shown to be safe in human trials (31, 68), a potential area of concern in their application as a vehicle for vaccines is the pre-existing immunity in the human population due to previous exposure from natural infections, in particular to Ad. It has been reported that the transgene product-specific Ab response was completely inhibited in humans after the administration of an AdHu5 vaccine vector (69), even with moderate titers of pre-existing NAb against AdHu5. A high prevalence of AdHu5-specific NAb was detected in both Gambian (84.67%) and South African (79.87%) populations (70). Nonetheless, in a phase 2 trial of AdHu5 vector-based Ebola vaccine in Sierra Leone, it was shown that a vaccine dose of $8.0\times10_{10}$ viral particles was safe and highly immunogenic in healthy Sierra Leonean adults, inducing specific Ab responses from day 14 onwards, which peaked at day 28, but declined quickly in the following months (71). Thus, to maintain Ab responses against the transgene, we have shown that an AAV1 boost might be a solution. AAV1 has lower seroprevalence compared with AAV2, the prototype of AAV (72). Moreover, it has minimal cross-reactivity against pre-existing NAbs against AAV2 (73). The seroprevalence against other serotypes of AAV, such as AAV5, AAV6, or AAV8, are even lower; thus, the development of malaria vaccines based on other serotypes will be an exciting future goal (74).

Furthermore, in this study, we demonstrated a generation of AdHu5- and AAV1-based multi-stage malaria vaccines harboring the gene encoding PfCSP fused with the Pfs25. Similar to the AdHu5-AAV1 Pfs25, the AdHu5-AAV1 Pfs25-PfCSP evoked high level of Ab titers against both Ags that are sustained for at least one transmission season, the desired ideal feature of malaria vaccine (13). Besides, the vaccines achieve both protection and TB immunity in a murine model, fulfilling the urgent need for an effective second-generation malaria vaccine which reduces transmission and incidence, rather than merely reducing

morbidity and mortality of the disease (75, 76). Moreover, it may meet the set strategic goals of the malaria vaccine technology roadmap by 2030 (1).

The Malaria Eradication Research Agenda Consultative Group on Vaccines (malERACGoV) required that a VIMT must primarily mitigate malaria transmission (12). A highly effective PEV that prevents erythrocytic stage infection will obviously reduce transmission. However, as previously mentioned, RTS,S/AS01 confers only 36.3% protection (77, 78). On the other hand, it has been suggested that a successful TBV would ideally be combined with a PEV or a blood stage vaccine (79). Hence, mixing both PEV and TBV might be a solution to achieve an effective VIMT. This regimen may also increase the adherence to the vaccination program for malaria elimination as people who get vaccinated will prefer a shot that also provides protection from the disease. A recent study has investigated the potential of mixing the RTS,S/AS01 with Pfs25-IMX313/AS01 in one formulation or co-administering both vaccines (21). The authors found that the combination of both vaccines elicited similar Ab titers against both PfCSP and Pfs25 as that of the single-Ag vaccines. Using in vitro assays, they also showed that the combination of vaccines exhibited similar functional activity in transmission blocking and sporozoite inhibition. Using in vivo assays, our result of the mixture of single-Ag formulation has been in line with this study, contrasting with several other studies demonstrating immune interference or reduced efficacies of the combination of several malaria vaccines (17, 80, 81).

However, mixing two or more vaccines in one formulation will result in higher vaccination cost. Development of a multi-valent vaccine harboring different Ags from different stages of parasite might be the best solution to reduce the vaccination cost. Addressing this issue, we employed the multi-stage AdHu5-AAV1 Pfs25-PfCSP and demonstrated similar efficacies of this regimen as those of the mixture of single-Ag formulation in both protection and transmission blocking. More remarkably, this multi-stage vaccine regimen exhibited a long-term transmission blocking, up to 9 months, with a sustained high titer of antibodies against Pfs25 exceeding one transmission season. These results are in contrast with previous development of multi-stage malaria vaccines demonstrating generally poor Ab responses (22-25). In the current study, we have focused on the pre-erythrocytic Ag, rather than blood-stage Ag, to combine with the mosquito-stage Ag. This combination might be the most efficient in reducing malaria prevalence as shown in a malaria model analysis of pathogen virulence evolution predicting that blood-stage vaccines select for higher virulence, while PEVs select for lower parasite virulence, which may increase the population-level benefits of vaccination (82) .

To conclude, AAV1 is a potential viral vector for PfCSP, Pfs25, and the fusion of both Ags as a booster vaccine following Ad-prime. With a 99% TRA, the AdHu5-AAV1 Pfs25 and AdHu5-AAV1 Pfs25-PfCSP vaccination regimen appear to be promising tools for achieving malaria eradication, as it has been shown that even a TRA of only 32% could reduce the basic reproduction number of the parasite by 20% and eliminate *Plasmodium* from mosquito and mouse populations at low transmission intensities in a laboratory model (83).

Future studies should be directed to investigate the long-term protective efficacy of the AdHu5-AAV1 Pfs25-PfCSP. However, even with lower protective efficacy, this regimen will be a great tool in supporting malaria elimination program as it has been shown that anti-sporozoite and anti-transmission interventions powerfully act synergistically to accelerate malaria elimination efforts over multiple generations (15). Moreover, additional TB immunity to the pre-erythrocytic immunity conferred by this multi-valent regimen is an improvement to the RTS,S/AS01 for next-generation vaccine (84).

Thus, these immunization regimen deserve further evaluation in clinical trials, where it can be used without safety concerns because Ad and AAV have been previously applied in humans as vaccine and gene therapy vectors, respectively.

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