Study on protection and transmission-blockade induced by multistage malaria booster vaccine based on Adeno-associated Virus serotype 5 (AAV5)

アデノ随伴ウイルス５血清型をベクターとしたマルチステージマラリア追加免疫ワクチンの感染防御及び伝播阻止両効果についての研究

Ammar Abdurrahman Hasyim

July 2022

Dissertation

Study on protection and transmission-blockade induced by multistage malaria booster vaccine based on Adeno-associated Virus serotype 5 (AAV5)

Graduate School of Medical Sciences

Kanazawa University

Division: Pharmaceutical Sciences

Laboratory: Vaccinology and Applied Immunology

School Registration Number : 1929012011

Name : Ammar Abdurrahman Hasyim

Primary Supervisor : Prof. Shigeto Yoshida, Ph.D.

SUMMARY

A leading malaria vaccine RTS, S/AS01 is the first malaria vaccine to be recommended to use for children in sub-Saharan Africa with moderate to high *P. falciparum* malaria transmission, although it has only modest efficacy and short durability, and it must be administered in a four-dose schedule to achieve high efficacy. The development of more efficacious vaccines is still needed. Viral vectored vaccines have a key advantage over protein-in-adjuvant vaccines like RTS, S, because they are capable of inducing cytotoxic CD8+T cell responses that are critical for the elimination of intracellular pathogens like malaria parasites.

Adeno-associated virus (AAV) is being utilized as an attractive vehicle for delivering genes to various target cells and tissues. It has been shown to induce efficient and long-term transgene expression with minimum level of toxicity in clinical trials. Very recently, we have shown that boosting with adeno-associated virus serotype 1 (AAV1) can induce highly effective and long-lasting protective immune responses against malaria when combined with a replication-competent vaccinia virus in a rodent model. In this study, we compare the efficacy of AAV5 with AAV1, as malaria booster vaccine following priming with vaccinia virus LC16m8Δ (m8Δ) strain, harboring a fusion gene encoding the pre-erythrocytic stage protein, *Plasmodium falciparum* circumsporozoite (PfCSP) and the transmission-blocking sexual stage (Pfs25). The two-dose heterologous prime-boost immunization regimen with m8Δ/AAV5 induce robust anti-PfCSP and anti-Pfs25 antibodies equivalent to m8Δ/AAV1. Regarding the protection, m8Δ/AAV5 achieve 100% sterile protection against malaria sporozoite challenge, compared with 70% protection of m8Δ/AAV1 vaccine. In contrast, the second challenge test with protected mice from 1st challenge showed m8Δ/AAV5 vaccine group provided 55.6% protection while 100% sterile protection of m8Δ/AAV1 vaccine group. Regarding the

transmission blockade, both m8Δ/AAV5 and m8Δ/AAV1 induced high level of the transmission-reducing activity (TRA: > 99%) and transmission-blocking activity (TBA: > 95%).

Our data indicate that AAV5-based boosting vaccine is an efficacious multistage malaria vaccine as good as AAV1-based one when administered following an m8Δ-based vaccine. These results suggest that AAV5 is an alternative vaccine vector if high levels of pre-existing anti-AAV1 antibody prevents re-immunization with the same serotypes and transgene expression.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AAV	Adeno-associated virus
AAV1	Adeno-associated virus serotype 1
AAV1-Luc	Adeno-associated virus serotype 1 expressing luciferase
AAV5	Adeno-associated virus serotype 5
AAV5-Luc	Adeno-associated virus serotype 5 expressing luciferase
AAV8	Adeno-associated virus serotype 8
Ab	Antibody
Abs	Antibodies
AdHu5	Human adenovirus type 5
Ag	Antigen
BSA	Bovine Serum Albumin
BV	Baculovirus
CSP	Circumsporozoite protein
DAPI	4’,6-diamidino-2-phenylindole
DFA	Direct-feeding assay
ELISA	Enzyme-linked immunosorbent assay

FITC	fluorescein isothiocyanate
HEK	Human Embryonic Kidney
HIV	Human Immunodeficiency Virus
HRP	Horse-radish peroxidase
ICS	Intracellular Cytokine Staining
IFAs	Immunofluorescence Assays
IgG	Immunoglobulin G
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
i.n.	intranasal
IVIS	*in vivo* imaging system
kDa	kilodaltons (protein size)
LC16m8	Japanese replication competent Vaccinia virus strain used for smallpox vaccination
LC16m8Δ	Highly attenuated and genetically stable variant of LC16m8
m8Δ	Simple abbreviations of LC16m8Δ
mAb	monoclonal antibody
MOI	Multiplicity of Infection

NAb	Neutralizing Antibody
NGS	Normal Goat Sera
Pb	*Plasmodium berghei*
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffer Saline containing 0.1% tween-20
pCAG	cytomegalovirus (CMV) enhancer fused to the chicken beta-actin promoter
pCMV*ie*	cytomegalovirus immediate early promoter
PE	pre-erythrocytic
PEV	Pre-erythrocytic Vaccine
Pf	*Plasmodium falciparum*
Pv	*Plasmodium vivax*
PfCSP	*Plasmodium 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
Pfs230	*P. falciparum* P230 protein that is expressed on gametocytes
Pfs25	*P. falciparum* P25 protein that is expressed on the surface of zygote and ookinete forms of malaria parasites.
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
RPE	R-Phycoerythrin
R.T.	Room Temperature
RLU	Relative Luminescence Units
s.c.	scarification
SD	Standard Deviation
SEM	Standard Error Mean
Spz	Sporozoite
TB	Transmission-blocking
TBA	Transmission-blocking activity
TBV	Transmission-blocking Vaccine
TRA	Transmission-reducing Activity
t.s.	tail scarification
vg	viral genome
VSV-G	vesicular stomatitis virus G glycoprotein

VV	Vaccinia Virus
WGCF	Wheat germ cell-free
WHO	World Health Organization

INTRODUCTION

Malaria is a primary mosquito-borne infection with deleterious effects on global health (1). The World Health Organization (WHO) reported 241 million malaria cases globally, leading to more than 600,000 deaths in 2020. This report reflects about 14 million more cases and 69,000 more deaths in 2020 than in 2019 (2).

Vaccination is one of the cost-effective measures for eradicating infectious diseases (3). Despite decades of effort, an effective malaria vaccine has remained unavailable. The ideal malaria vaccine should be able to stimulate protective immune responses while also inhibiting parasite transmission from mosquitoes to humans (4, 5). A leading malaria vaccine, RTS, S/AS01, is the first vaccine recommended for children in sub-Saharan Africa. However, it has only modest efficacy and short durability. Phase 3 clinical trial demonstrated that the vaccine efficacy was 36% over 4 years of follow-up among children aged 5-17 months. It must be administered in a four-dose schedule to achieve high efficacy (6, 7). On the other hand, only three transmission-blocking vaccines (TBV) candidate antigens – Pfs25 (*P. falciparum*), Pvs25 (*P. vivax*), and Pfs230 (*P. falciparum*) – have undergone human clinical testing. One of these, Pfs25, induced TRA antibody in phase 1 clinical studies (8-11). Pfs25 does not appears to induce naturally occurring antibodies in malaria-endemic areas (12). It has stage-specific surface expression of zygotes and ookinetes in the mosquito vector (13, 14). Therefore, those two facts provide reasons to develop a more effective malaria vaccine.

A vaccine based on viral vectors offers excellent potential for solving the obstacles to malaria elimination. It has a crucial advantage over protein-in-adjuvant vaccines. It can induce cytotoxic $CD8^+$ T cell responses that are critical for eliminating intracellular pathogens like malaria parasites (15, 16). Among the available viral gene delivery systems, adeno-associated

viruses (AAVs) are appealing vaccine vector candidates (17-19). AAVs can target a particular tissue or cell type by using a specific serotype (20, 21). To date, more than hundreds of AAV variants have been examined in various model strategies (22, 23). Thus, AAV is the good candidate for the malaria vaccine.

AAV1 has reported transducing muscle cells (24-26). AAV1 harboring *Plasmodium falciparum* circumsporozoite protein (PfCSP) or the transmission-blocking sexual stage P25 protein (Pfs25) antigen achieved a high level of protection against sporozoite challenge (27). It also could achieve excellent transmission-blocking (TB) activity with sustained high-level antibody responses following human adenovirus serotype 5 (AdHu5) priming (27). Recently we established that an AAV1-based vaccine could induce robust immune responses, durable and high-level protection, and TB efficacy when initially primed with an LC16m8Δ (m8Δ). A replication-competent vaccinia virus (VV), LC16m8, has been administered to 100,000 Japanese children without severe adverse event (28). As a genetically stable variant of the LC16m8 vaccine (29), the m8Δ induced about 500 times more efficient of immune responses than the replication-deficient Vaccinia virus (29).

The presence and variation in pre-existing immunity to AAV serotypes suggest that no single AAV serotype is generally relevant for viral vector platform (30). Thus, it also important to utilize and develop alternate AAV serotypes for malaria vaccine candidate. AAV5 has been characterized as the serotype with the lowest seroprevalence of pre-existing neutralizing factors (31). It was a significant advantage versus other AAV serotypes in overcoming the barrier of pre-existing antibodies (32, 33). A systemic administration of AAV5 delivers copies of the human factor IX (hFIX) gene to the liver (AAV5-hFIX) successfully treated hemophilia B patients in the presence of detectable pre-existing anti-AAV5 neutralizing antibodies (34, 35). Furthermore, AAV5 has been reported to transduce muscle, liver, and lung tissue (36-39). An administration of AAV5 harboring human papillomavirus protein L1 (HPV-L1) could induce

a high level of L1-specific immune responses in mice and rhesus macaques (40). An AAV5-based therapeutic vaccine harboring *neu oncogene* administration could confer a 50% survival rate against challenges with TUBO breast cancer cells over 100 days (41).

The present study aimed to compare AAV5-based with AAV1-based vaccine as malaria multistage vaccines when initially primed by the m8Δ-based vaccine. In pursuit of this aim, we examined the transduction efficiency in a mammalian cell line, the ability to induce malaria functional antibodies, the protective efficacy, and the transmission-blocking efficacy of both immunization regimens. Our result supports the further evaluation of the AAV5-based vaccine as an alternative candidate to AAV1-based vaccine if high levels of pre-existing antibody prevent re-immunization with the same serotypes and transgene expression.

AIMS OF THE STUDY

To compare the efficacy of AAV5- with AAV1-based vaccine as a multi-stage malaria booster vaccine when initially primed by Vaccinia virus, LC16m8Δ vaccine. In addition, the specific aims of the study were:

1. To compare the transduction ability of AAV5- with AAV1-based vaccine drive malaria antigens in a mammalian cell line.
2. To examine whether the AAV5 or AAV1 vectors could induce long-term luciferase transgene expression in murine model by intramuscular or intravenous administration.
3. To check the humoral immune responses induced by AAV5- or AAV1-based malaria vaccine when initially prime with m8Δ-based vaccine.
4. To compare the protective efficacy of prime-boost immunization regimen using m8Δ/AAV5-Pf(s25-CSP) with m8Δ/AAV1-Pf(s25-CSP).
5. To assess the transmission-blocking efficacy of vaccine regimens using m8Δ/AAV5-Pf(s25-CSP) or m8Δ/AAV1-Pf(s25-CSP).

MATERIALS AND METHODS

Ethics Statement

All animal protocols and handling procedures were approved by the Animal Care and Use Committee of Kanazawa University (No.AP-214212). All precautions were taken to minimize the suffering to the animals throughout the study.

Parasites and Animals

The transgenic parasite, *P. berghei* expressing PfCSP (PfCSP-Tc/Pb) was used for the protective efficacy study and Pfs25DR3 (PbPfs25DR3) was used for TB assays as described previously (26, 42, 43). Both transgenic parasites were maintained in the Laboratory of Vaccinology and Applied Immunology, Kanazawa University. Six-week-old female ddY and BALB/c mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). BALB/c mice were used to assess immune responses, protective efficacy, and transmission blocking assay. *Anopheles stephensi* mosquitoes (SDA 500 strain) were infected with the transgenic parasites by allowing them to feed on parasitized 6-week-old ddY mice.

Viral Vector Construction

For the generation of m8Δ-Pf(P7.5-s25-CSP)-HA, BHK-21 cells were infected with canarypox virus, and then transfected with the transfer vector encoding the Pfs25–PfCSP fusion protein and purified genomic DNA of LC16m8Δ2. RK13 cells were exposed to the lysates of transfectants to develop plaques, and then incubated with the peripheral blood of a white leghorn chicken. For the generation of AAV1-Pf(s25-CSP) or AAV5-Pf(s25-CSP), the gene cassette encoding 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. The resulting plasmid, pAAV-CMV-sPfs25-sPfCSP2, was used to generate

AAV1-Pf(s25-CSP) or AAV5-Pf(s25-CSP) by transfecting HEK293 cells, as described elsewhere (27, 44). AAV1 or AAV5 expressing luciferase (AAV1-Luc or AAV5-Luc) were generated as described previously (45, 46).

In Vivo Bioluminescence Imaging

On day 0, AAV1 or AAV5 expressing luciferase (AAV1-Luc or AAV5-Luc) were administered into the right medial thigh muscles or tail veins of the BALB/c mice (n = 3; 1.0 x 10^{11} viral genomes [vg]/mouse. The mice were injected with a ketamine (100 mg/kg)/xylazine (10 mg/kg) mixture for anesthesia. Then, 10 minutes later, D-Luciferin (15 mg/mL; OZ Biosciences, Marseille, France) was administered intraperitoneally (i.p.) 150 ul/mouse at the appropriate time points. Luciferase expression in whole bodies was detected using IVIS® Lumina LT *in vivo* imaging system (PerkinElmer, Waltham, MA, USA) as described previously (45, 47).

Immunoblotting

To measure PfCSP and Pfs25 protein expression in mammalian cell lines infected with viral vector vaccine regimen, HEK293T cells were infected with LC16m8Δ vaccine at a multiplicity of infection (MOI) of 5 per 1x10^6 cells or transduced with the AAV1 or AAV5 vaccines at an MOI of 10^5 per 4x10^4 cells. Cells were lysed by Laemmli buffer at 24 hours for LC16m8Δ-infected cells and 48 hours for AAV-infected cells and subjected to immunoblotting, as described previously (45). The cell lysates were separated and electrophoresed onto 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels under reducing conditions for PfCSP and non-reducing conditions for Pfs25.

Proteins were then transferred to Immobilon FL®PVDF membranes (Merck Millipore, Tokyo, Japan), using a wet transfer apparatus (BioRad lab, Inc., Tokyo, Japan). The membranes were blocked in 5% skim milk in PBS at room temperature (R.T.) for 1 hour

containing 0.1% Tween 20 (PBS-T) and incubated overnight with the monoclonal antibody (mAb) anti-PfCSP 2A10 diluted 1:10,000 or anti-Pfs25 4B7 diluted 1:2,000 together with anti-GAPDH diluted 1:10,000 in 5% skim milk at 4^{o}C. The blots were then washed three times with PBST and probed with the secondary antibody, goat anti-mouse conjugated to IRDye 800 (Rockland Immunochemicals, Limerick, PA, USA), diluted 1:20,000 in 5% skim milk at R.T. for 1 hour. The membrane was then visualized on an Odyssey infrared imager (LI-COR, Lincoln, NE, USA), according to the manufacturer's protocol. The strength of band intensities was analyzed using ImageJ Java-based image processing program (LOCI, University of Wisconsin).

Immunofluorescence Assay

To assess the protein expression and localization in a mammalian cell line, the immunofluorescence assays (IFAs) were performed as described previously (26). HEK293T cells were infected with the m8Δ at an MOI of 0.1 or transduced with AAV1 or AAV5 on eight-well-chamber slide at an MOI of 10^5 for 1 hour and then incubated in growth media for 8 hours for m8Δ or 24 hours for AAV1 and AAV5. The cells were then fixed with 100% methanol (permeabilized) at -20^{o}C or 4% paraformaldehyde (non-permeabilized) at R.T. for 15 minutes. Then, to visualize the expression of proteins, the cells were washed with ice cold PBS (-) and stained with R-Phycoerythrin (RPE)-conjugated 2A10 and fluorescein isothiocyanate (FITC)-conjugated 4B7, diluted 1:200 or 1:100, respectively, in 5% normal goat serum (NGS)/PBS for 1 hour at R.T. VECTASHIELD containing 4`,6-diamidino-2-phenylindole (DAPI) was used for nuclear staining. Images were taken using a BZX710 fluorescence microscope (Keyence Corp, Tokyo, Japan) or confocal laser scanning microscope (CLSM). The positive cells were count individually and percent (%) positive cells that either induced by AAV5 or AAV1 were calculated as follows: 100% x (the number of positive cells / the total number of cells).

Immunization

Six-week-old female BALB/c mice were housed in the Animal Facility of Kanazawa University for at least 1 week prior to vaccination experiments. We primarily used three regimens of two-dosage prime/boost immunization. All groups were primed by Vaccinia virus, m8Δ-Pf(P7.5-s25-CSP)-HA by a tail scarification (t.s.) at the dose of $1x10^7$ plaque forming unit (PFU)/animal using truncated needle on the one-third proximal tail. Then, 6 weeks later, 10 of mice in separated groups boosted by intramuscular (i.m.) inoculation of AAV1- or AAV5-Pf(s25-CSP) vaccination regimens into the right hindlimb at the dose of $1x10^{11}$ vg/animal, respectively. As immunization controls, PBS was used for both priming and boosting administration.

Enzyme-Linked Immunosorbent Assay

The PfCSP- or Pfs25-specific antibody (Ab) levels were quantified by ELISA as described previously (26, 45). The PfCSP protein was purified using an *Escherichia coli* expression system and the Pfs25 protein was produced using a wheat germ cell-free (WGCF) protein expression system (Cell Free Sciences, Matsuyama, Japan)(13). Sera from immunized mice were collected from tail vein blood samples 1 day before boost and 1 day before challenge.

PfCSP or Pfs25 proteins were coated onto a 96-well plate (Costar EIA/RIA polystyrene plates, Corning Inc., NY, USA) for 24 hours at 4°C. The plates were then blocked with 1% bovine serum albumin (BSA) in PBS solution for 1 hour. Coated plates were incubated for 1 hour at R.T. with serum samples as well as with negative and positive controls mAb 2A10 or 4B7 that had been serially diluted in PBS containing 1% BSA, then washed with 3x PBST, 1x PBS and then, incubated with secondary Ab, horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Bio-Rad) diluted 1:2000 in PBS containing 1% BSA at R.T. for 1 hour. The endpoint titer is 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.

Parasite Challenge Test

Six or 14 weeks after the last immunization, the mice were challenged with an intravenous (i.v.) of PfCSP-Tc/Pb sporozoites resuspended in RPMI 1640 media (Gibco, Life Technologies, Tokyo, Japan). Sporozoites were prepared as described previously (48). Each mouse was injected via the tail vein with 100 µl of media containing 2,500 sporozoites. Infections were monitored from day 4 to 14 using the Giemsa staining of thin blood smears obtained from the tail. At least 20 fields (magnification: x1,000) were examined before a mouse was deemed to be malaria-infection negative. Protection was defined as the complete absence of blood-stage parasitemia on day 14 post-challenge. The time required to reach 1% parasitemia was determined as described previously (49).

TB Assays

TB was assessed using direct-feeding assays (DFAs) as described previously (26). At 35 days after boost, the mice were treated with phenyl hydrazine (PHZ) and infected i.p. with 10^6 *P. berghei* Pfs25DR3-parasitized red blood cells (pRBCs) 3 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 hours 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^{o}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 of oocyst intensity was calculated. For inhibition calculations, these numbers were compared with those of mice without immunization. Percent (%) inhibition of mean oocyst intensity (transmission-reducing activity; TRA) was calculated as follows: 100 x [1 –

(mean number of oocysts in the test group/ mean number of oocysts in the control group)]. In addition, the percentage of oocyst prevalence inhibition (transmission-blocking activity (TBA)) was evaluated as: 100 x [1 – (proportion of mosquitoes with any oocysts in the test group)/(proportion of mosquitoes with any oocyst in the control group)] (50).

Statistical Analysis

The statistical analysis for evaluating significantly results between groups was performed by GraphPad Prism version 8.0 for Mac OS. Man-Whitney *U*-test was used for analyzing differences between two groups. An unpaired *t*-test with Welch's correction was used for comparing groups with unequal sample sizes. All ELISA end-point titers were $\log_{10}$-transformed before analysis. The proportion of mice not reaching 1% parasitemia was analyzed using a Kaplan-Meier log-rank (Mantel-Cox) test.

RESULTS

In Vitro Transduction Efficiency of AAV5 in A Mammalian Cell Line

The malaria fusion gene, *pfs25-pfcsp*, was linked by a hinge peptide (Gly6Ser) between the *pfcsp* and *pfs25* genes, and its expression was driven by the CMV immediate-early enhancer-promoter (CMV*ie*) for AAV1 or AAV5 and 7.5 promoter (P7.5) for m8Δ **(Figure 1A)**. Our previous study showed that a fusion protein Pfs25-PfCSP in the cells transduced with AdHu5 or AAV1 were visualized with both mAb 2A10 (anti-PfCSP) and 4B7 (anti-Pfs25) as a ladder of bands of 80-100 kDa (27).

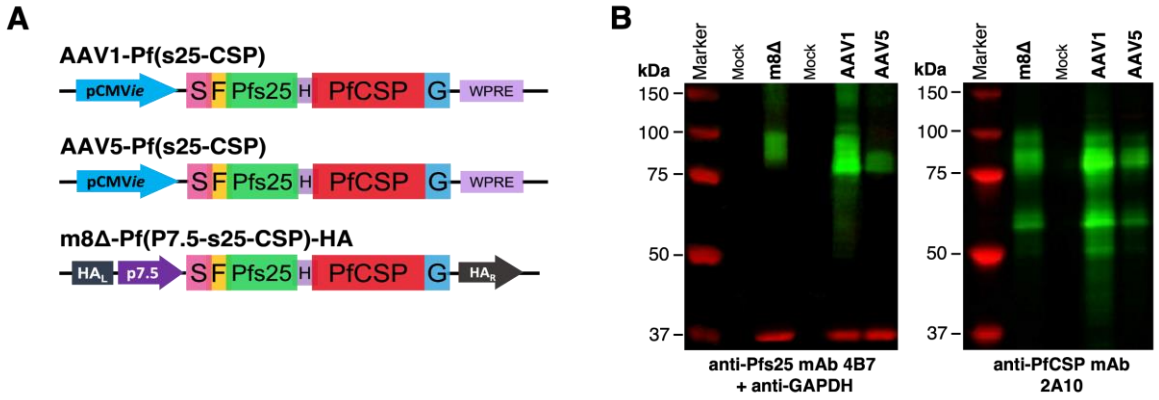


Figure 1: Expression of Pfs25 and PfCSP transduced by m8Δ and AAVs. (A) Construction of viral-vectored vaccines. (B) Expression of the Pfs25 and PfCSP in HEK293T cells transduced or not (mock) by m8Δ-Pf(s25-CSP)-HA (MOI = 5) or AAV1- or AAV5-Pf(s25-CSP) (MOI = 10^5). Cell lysates were loaded onto 10% SDS-PAGE gels and immunoblotted with anti-PfCSP mAb 2A10 (green) or anti-Pfs25 mAb 4B7 (green) and anti-GAPDH (red). pCMV*ie* : Cytomegalovirus immediate-early enhancer-promoter, S : the mouse IgGκ signal peptide, F : FLAG tag, H : Hinge peptide (Gly6Ser), G : GPI anchor, WPRE : Woodchuck Hepatitis Virus (WHV) Post-transcriptional Regulatory Element.

Likewise, in the present study, similar results were obtained with m8Δ (MOI = 5, lane 1) or AAV1 or AAV5 (MOI = 10^5, lane 2 and 3) also reacted with both mAb 2A10 (anti-PfCSP) and 4B7 (anti-Pfs25) as a ladder band with relative *Mr* of 80-100 kDa (**Figure 1B**). The Pfs25-

PfCSP fusion protein in the cells infected with AAV1 reacts 3.4 and 4 times stronger with both mAb 2A10 and 4B7, respectively, as compared to those in the cells infected with AAV5.

An IFA analysis showed that Pfs25 and PfCSP epitopes were expressed both in the cytoplasm and on the surface of infected cells after m8Δ (**Figure 2A**) and AAVs transduction (**Figure 2B, C**). Interestingly, the localization and distribution of fusion protein induced by AAV5 was about 7.6 times lower than that expression induced by AAV1 (**Figure 2B**).

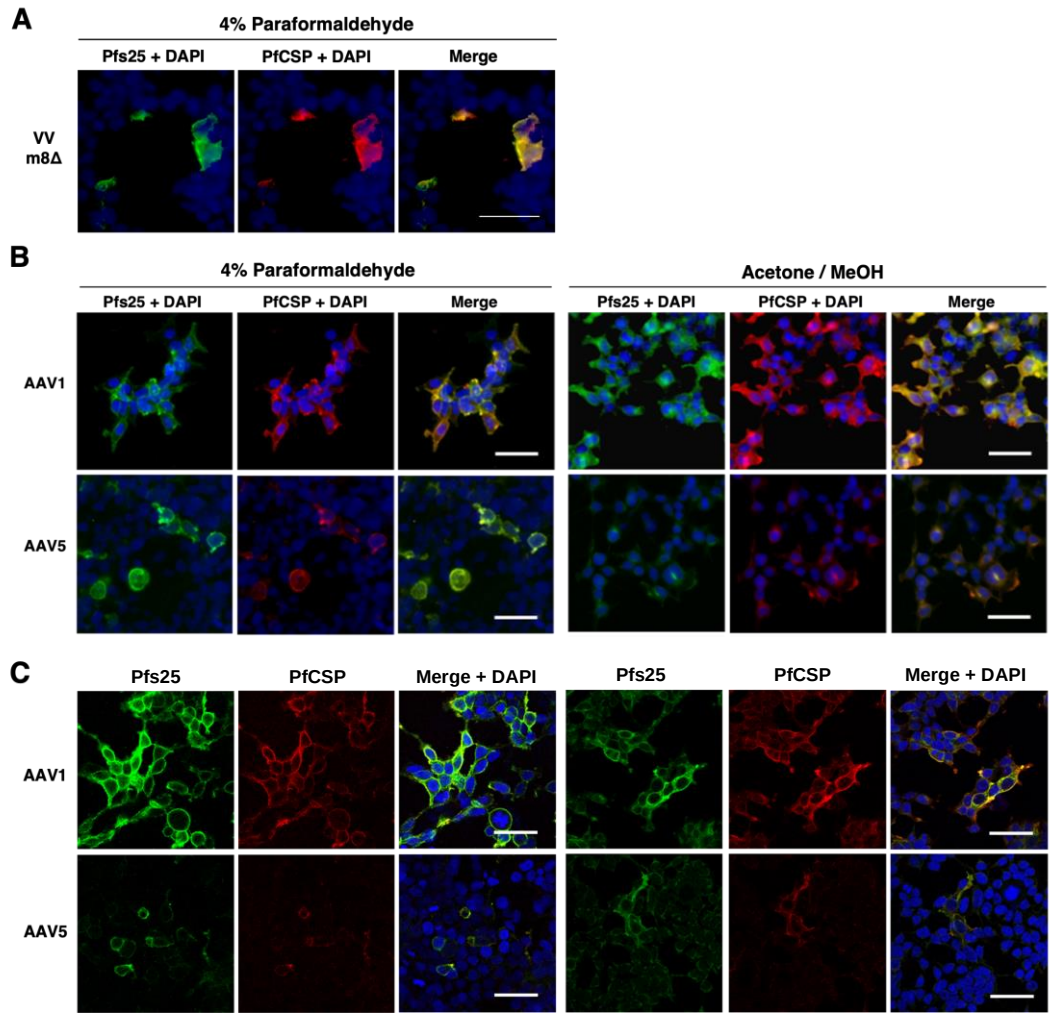


Figure 2 | Localization and Distribution of PfCSP and Pfs25 expression in mammalian cells. (A-C) HEK293T cells transduced by m8Δ (MOI = 0.1) (A), or AAV1- or AAV5-Pf(s25-CSP) (MOI = 105) (B, C). Images were taken using a BZX710 conventional fluorescence microscope (A, B) and confocal microscope (C) for an optical section of the cells as determined by IFA. After 8 hours (A) or 24 hours (B, C), cells were fixed with methanol (permeabilized) or 4% paraformaldehyde (non-permeabilized) and blocked with 5% normal goat sera. After being blocked, the cells were incubated with FITC-conjugated mAb 4B7 (green) and R-PE-conjugated 2A10 (red). Cell nuclei were visualized with DAPI (blue). Original magnification, 400x. Scale bars = 50 μm.

The number of positive cells that either induced by AAV5 and AAV1 were count individually (105/903 (11.6%) and 863/978 (88.2%); respectively). The similar pattern was confirmed with an optical section of the cells using a confocal microscope (**Figure 2C)**. Although the AAV5 vector induced a lower protein expression than AAV1 did, the fusion protein was still expressed on the surface of the transduced cells (**Figure 2B, C left panel**).

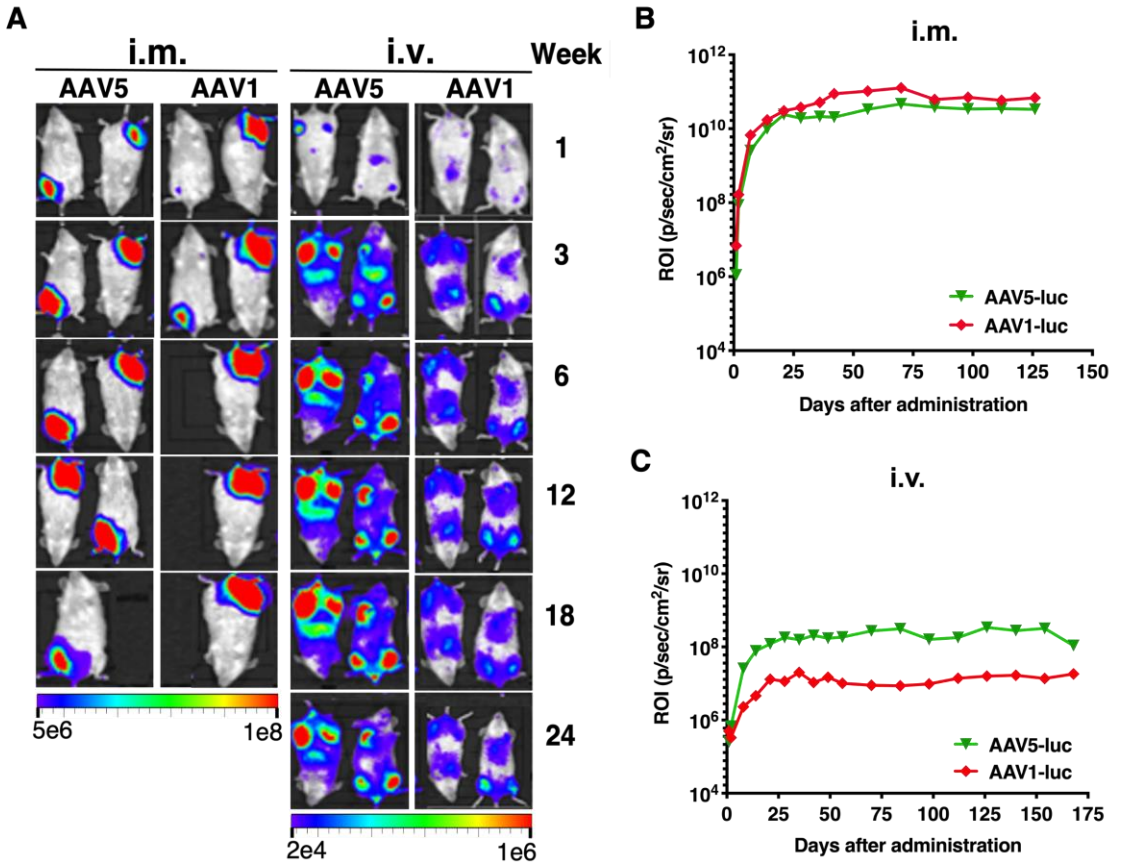


Figure 3 | Long-term Transgene Expression of AAV5-Luc and AAV1-Luc. (A) AAV5-Luc or AAV1-Luc was injected into the tail veins or right medial thigh muscles of BALB/c mice (n = 2; 1.0×10^{11} vg/mouse) on day 0. Luciferase expression at different time points was detected using the IVIS Lumina LT Series III *in vivo* imaging system. The heatmap images visible in the mice represent the total flux of photons (p/s/cm^2) in the area of interest. Rainbow scale ranges are expressed in radiance (p/s/cm^2/sr). (B, C) The mean of total flux of photons in the region of interest (ROI) right hind leg from day 0 to 126 (18 weeks) after i.m. (B) or whole body from day 0 to 168 (24 weeks) after i.v. (C) AAV-Luc administration is shown.

AAV5 Exhibits Long-term and High-level of Luciferase Expression by i.m. Administration

To examine the efficient transduction and biodistribution of AAV5 or AAV1 *in vivo*, AAV5 or AAV1 expressing luciferase, AAV1-Luc or AAV5-Luc was administered i.m. into the right medial thigh muscle of BALB/c mice or i.v. into the tail vein (10^{11} vg/ mouse, $n = 3$) on day 0. The luminescence signal after i.m. administration of AAV5-Luc or AAV1-Luc achieved the robust luciferase expression in the right hind leg (**Figure 3A**). It gradually increased from day 0 to day 21 and persisted for over 126 days with the peak of total flux of 2.42 x 10^{10} and 3.06 x 10^{10} photons/s/cm^2/sr induced by AAV5-Luc and AAV1-Luc, respectively (**Figure 3B**). The luciferase activity levels did not significantly differ between these vectors. This result indicates that our AAV5 or AAV1 vector system were more efficiently transduced muscle cells and exhibited the long-term transgene expression, as shown in previous studies (26, 51). Conversely, the luminescence signal after i.v. administration of AAV5-Luc and AAV1-Luc conferred the luciferase expression ~100 and ~1000 times lower than i.m. administration, respectively. The peak of total flux of 1.21 x 10^8 and 1.31 x 10^7 photons/s/cm^2/sr induced by AAV5-Luc and AAV1-Luc, respectively, at day 21 after AAVs vector administration. The peak of luminescence signal of both regimens persisted for up to 168 days (24 weeks) (**Figure 3A, C**). Based on the superiority of i.m. over i.v. route (**Figure 3A, B**) and the risk of unpredicting target bias of AAVs vaccine by i.v. delivery (**Figure 3A, right panel**), i.m. administration was decided to examine immunogenicity and protective efficacy.

Induction of Robust Humoral Immune Responses by m8Δ/AAV5-Pf(s25-CSP) Vaccination

The heterologous immunization regimen in mice was conducted by using the m8Δ-Pf(s25-CSP)-HA as a prime and AAV5-Pf(s25-CSP) as a boost. M8Δ/AAV1-Pf(s25-CSP) immunization was also conducted as a vaccination control. The result shows that either m8Δ/AAV5-Pf(s25-CSP) and m8Δ/AAV1-Pf(s25-CSP) immunization induced robust anti-

PfCSP IgG titers (2,265,556 and 2,714,900 after boosting, respectively) or anti-Pfs25 IgG titers (460,571 and 717,423 after boosting, respectively). We did not observe significant differences in Ab production, either anti-PfCSP or anti-Pfs25 induced by m8Δ/AAV5 or m8Δ/AAV1 (**Figure 3A, B).** These results indicate that m8Δ/AAV5 vaccine induced high-level PfCSP-specific humoral immune responses and Pfs25 functional Ab at comparable level to those of the m8Δ/AAV1 vaccine.

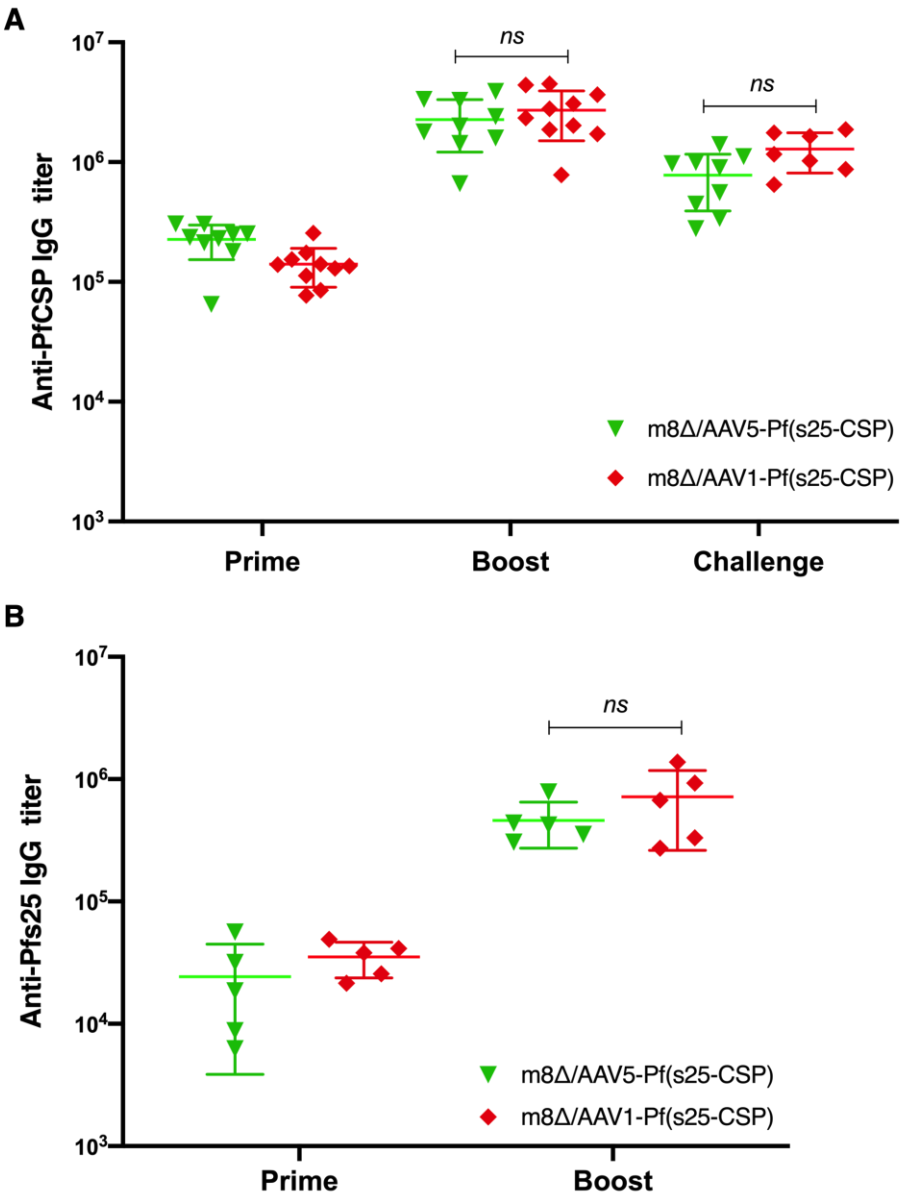


Figure 4 | Immunogenicity of the m8Δ/AAV5-Pf(s25-CSP) or m8Δ/AAV1-Pf(s25-CSP). BALB/c mice were immunized with the indicated regimen at a 6-week interval (*n* = 5 or 9 or 10). Individual sera were collected one day before boost and challenges. (A, B) Antibody responses induced by m8Δ/AAV5-Pf(s25-CSP) or m8Δ/AAV1-Pf(s25-CSP). Anti-PfCSP (A) and anti-Pfs25 (B) were measured by ELISA. Each symbol represents a single mouse. Differences between groups were assessed with Mann-Whitney *U*-test. *ns*, not significant.

The m8Δ/AAV5-Pf(s25-CSP) Confers Sterile Protection against Transgenic P. berghei Expressing PfCSP

To evaluate the protective efficacy of the m8Δ/AAV5-Pf(s25-CSP), immunized mice were exposed to two series of challenges with 2,500 PfCSP-Tc/Pb sporozoites at 40 days and 100 days post-boost, respectively. At day 40 after the last immunization, 9/9 mice (100%, $p < 0.0001$) were protected in the m8Δ/AAV5-Pf(s25-CSP) group, whilst 7/10 mice (70%, $p = 0.0031$) were protected in the m8Δ/AAV1-Pf(s25-CSP) group (**Table 1**) (**Figure 4A**).

Table 1 | Protective efficacies against sporozoites challenges by immunization with heterologous prime-boost regimens in a murine model [a]

Prime	Boost	1st Challenge [b, c]	2nd Challenge [b, c]
		Protected/ Challenged (% Protection)	Protected/ Challenged (% Protection)
PBS	PBS	0/10 (0)	0/10
m8Δ-Pf(P7.5-s25-CSP)-HA	AAV5-Pf(s25-CSP)	9/9 (100%)	5/9 (55.6%)
m8Δ-Pf(P7.5-s25-CSP)-HA	AAV1-Pf(s25-CSP)	7/10 (70%)	7/7 (100%)

[a] Mice were immunized with a scarification of $1x10^7$ pfu of Vaccinia virus, m8Δ-Pf(P7.5-s25-CSP)-HA followed by a booster with $1x10^{11}$ vg per mouse of AAV5-Pf(s25-CSP) administered i.m. at a 6-week interval.

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

[c] Significant difference from the PBS group as a control was determined using Fisher's exact probability test *(****$p < 0.0001$, **$p < 0.01$, *$p < 0.05$).*

At day 100 after the last immunization, 5/9 mice (55.6%, $p = 0.0108$) were protected in the m8Δ/AAV5-Pf(s25-CSP) group, whilst 7/7 mice (100%, $p < 0.0001$) were protected in the m8Δ/AAV1-Pf(s25-CSP) group (**Figure 4B**). The vaccination regimen m8Δ/AAV1-Pf(s25-CSP) induced significantly better protection against second exposure than did the m8Δ/AAV5-Pf(s25-CSP) regimen (100% vs 55.6%, $p < 0.05$) (**Figure 4B**).

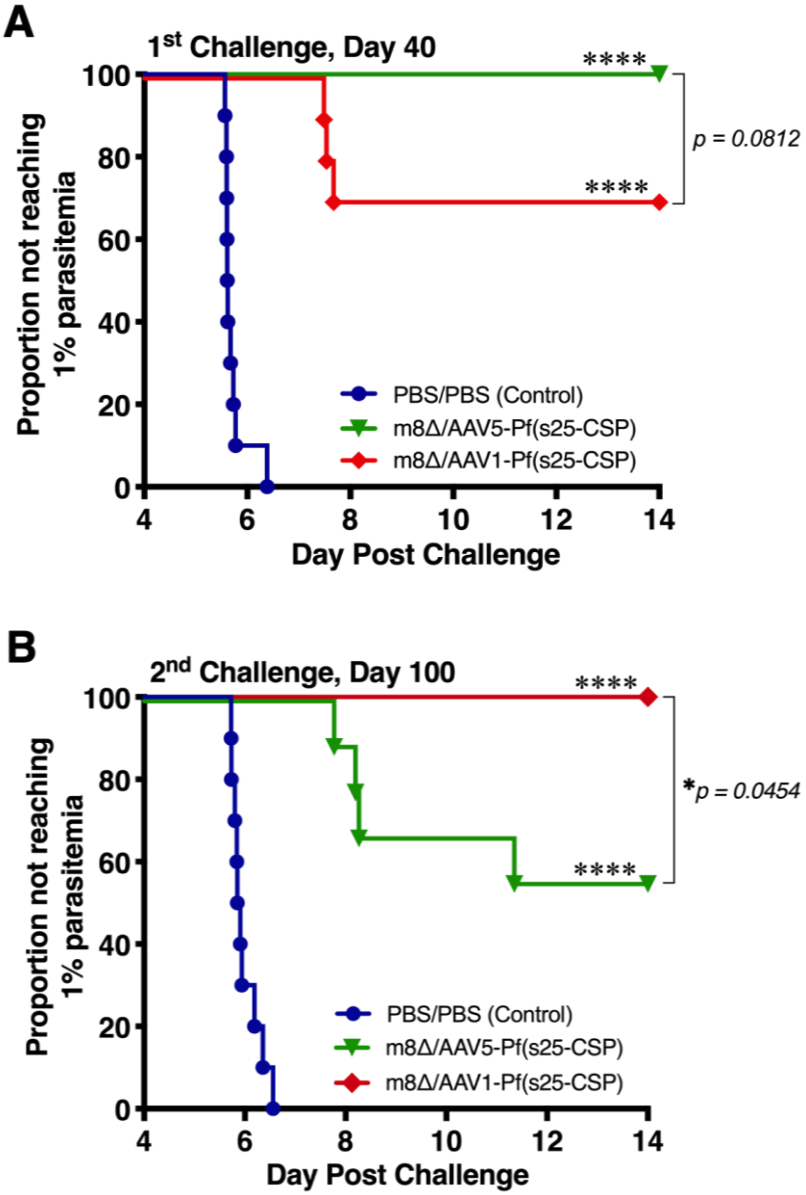
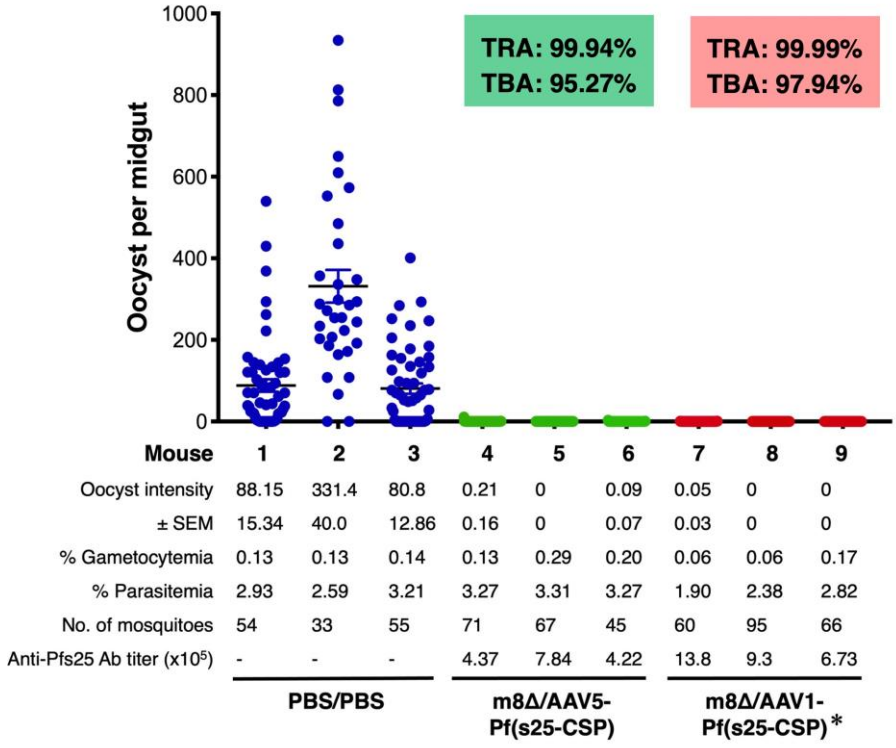


Figure 5 | Protective Efficacy. BALB/c mice (n = 9 or 10/ group) were immunized with indicated regimen at a 6-week interval. (A) Forty days after the boost, the mice were challenged with an i.v. injection of 2,500 transgenic PfCSP-Tc/Pb sporozoites. (B) Then, 100 days after the boost, survived mice from each group were rechallenged with 2,500 PfCSP-Tc/Pb sporozoites. Parasitemia was monitored for three consecutive days, starting from day four after the challenge, and a model predicting the time to reach 1% parasitemia was generated. The absence of blood-stage parasites in the animals was confirmed on day 14 post-challenge. The statistical analysis was performed by generating Kaplan-Meier survival curves, and *p*-values were calculated with Kaplan-Meier log-rank (Mantel-Cox) tests for vaccine groups versus PBS (control) group. Differences between vaccine groups were assessed by unpaired *t*-Test with Welch's correction. ****$p < 0.0001$, *and* *$p < 0.05$.

These results indicate that m8Δ-prime/AAV5-boost were still successfully eliminated twice exposures of high number parasites burden by induced PfCSP-specific humoral immune responses and protects against challenges by vaccine-specific adaptive immune responses.



Mouse	1	2	3	4	5	6	7	8	9
Oocyst intensity	88.15	331.4	80.8	0.21	0	0.09	0.05	0	0
± SEM	15.34	40.0	12.86	0.16	0	0.07	0.03	0	0
% Gametocytemia	0.13	0.13	0.14	0.13	0.29	0.20	0.06	0.06	0.17
% Parasitemia	2.93	2.59	3.21	3.27	3.31	3.27	1.90	2.38	2.82
No. of mosquitoes	54	33	55	71	67	45	60	95	66
Anti-Pfs25 Ab titer (x10^5)	-	-	-	4.37	7.84	4.22	13.8	9.3	6.73
	PBS/PBS			m8Δ/AAV5-Pf(s25-CSP)			m8Δ/AAV1-Pf(s25-CSP)*		

Figure 6 | TB Efficacy. BALB/c mice were immunized with the indicated regimen at a 6-week interval (n = 5). Three of the mice were bitten by mosquitoes in DFA. On days 10-12 post-feeding, the mosquitoes' midgut was dissected, and oocyst intensity and prevalence were determined. Each data point represents a single blood-fed mosquito. The x-axis columns represent individual mice. Horizontal lines indicate the mean number of oocysts observed [± standard errors of the means (SEM)].

The m8Δ-AAV5 s25-CSP Elicits a Potent TB Effect

A direct-feeding assay was conducted using Pfs25DR3-pRBCs 35 days after the last immunization (52). Mosquitoes that fed on the three control mice displayed an average intensity of 166.8 oocyst/midgut, while those that fed on the mice vaccinated with m8Δ/AAV5-Pf(s25-CSP) had a mean intensity of 0.1 oocysts / midgut; thus, the vaccination achieved significant transmission-reducing activity (TRA) of 99.94% ($p < 0.0001$) (**Figure 5**).

The percentage of infected mosquitoes was reduced from 80.91% in the control group to 3.82% in the vaccinated group, achieving significant TB activity (TBA) of 95.27% ($p < 0.0001$). Similarly, m8Δ/AAV1-Pf(s25-CSP) vaccination conferred TRA of 99.99% ($p < 0.0001$) and TBA of 97.94% ($p < 0.0001$). There were no significant differences in the TRA and TBA between the two groups ($p = 0.1107$ and $p = 0.1987$, respectively.

Table 2. Transmission-blocking activity of the prime-boost immunizations regimens

Group	Prime	Boost	Mean Intensity ± SEM (oocysts per midgut)	Mean Prevalence ± SEM (% infected mosquitoes)	TRA (%)[a,b]	TBA (%)[c,d]
Control	PBS	PBS	158.98(129.11)	80.91 (14.40)		
G.1	m8Δ-Pf(P7.5-s25-CSP)-HA	AAV5-Pf(s25-CSP)	0.100 (0.11)	3.82 (3.56)	99.94****	95.27****
G.2	m8Δ-Pf(P7.5-s25-CSP)-HA	AAV1-Pf(s25-CSP)	0.017 (0.03)	1.667 (2.89)	99.99****	97.94****

[a] Transmission-reducing activity (TRA) was calculated by comparing with the control group, and significant differences were assessed using a Mann-Whitney U-test (****$p < 0.0001$).

[b] No significant difference between the TRA of m8Δ /AAV1 and m8Δ /AAV5 vaccine regimens ($p = 0.1107$).

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

[d] No significant difference between the TBA of m8Δ /AAV1 and m8Δ /AAV5 vaccine regimens ($p = 0.1987$).

These results indicate that both vaccine regimens induced a high level of anti-Pfs25 IgG titer, which was enough to inhibit oocysts formation in mosquito's midgut. Thus, the AAV5-based vaccine is a promising transmission-blocking vaccine candidate which able to confer high-level and potent TB activity as good as AAV1-based vaccine.

DISCUSSION

The present study demonstrates that AAV5-based is efficacious malaria boosting vaccine as good as AAV1-based one when initially prime with m8Δ-based vaccine. It can induce robust humoral immune responses and sustained after the challenge against transgenic malaria parasites. Notably, it confers sterile protection after initial challenge with transgenic sporozoites expressing PfCSP. In contrast, AAV1-based is still a superior vectored vaccine when it can sustain complete protection of survived mice after the second malaria parasites challenge. Regarding the transmission blockade, both m8Δ/AAV5 and m8Δ/AAV1 induce high level of the TB efficacy.

Malaria vaccine based on AAV vectors could induce a high level of malaria-specific immune responses, protective efficacy, and transmission blocking activity in murine model (26, 53). An AAV1-based single or multistage malaria vaccines conferred potent and durable protection as well as TB efficacy when administered following an AdHu5 priming vaccine (26, 27). A liver directed pre-erythrocytic vaccine (PEV) based on AAV8 improved humoral and cellular immune responses and achieved sterile protection against sporozoites challenge (53). The most current study showed that m8Δ-prime/AAV1-boost achieved life-long sterile protection and high-level transmission blocking efficacy after multiple malaria parasites exposures. Here, we showed that the AAV5-Pf(s25-CSP) induced immunogenicity and protective efficacy as strong as AAV1-Pf(s25-CSP) did. This finding may provide an alternative AAV vector-based malaria vaccines because natural immunity to AAV5 is rare (38). In addition, AAV5 vectors has low seroprevalence of pre-existing neutralizing antibodies (54).

The sterile protection achieved by m8Δ/AAV5 vaccine after initial sporozoites challenge was due to the high level of Ab production and might be related with activation of cytotoxic T cell to eliminate intracellular parasites as well. AAV5 vectors can efficiently transduce

dendritic cells (DCs) and produce stronger antigen-specific immune responses than other AAV vectors (44). DCs are stimulators of T and B lymphocytes. It is mainly responsible for stimulating naïve T lymphocytes and initiating a cytotoxic T lymphocytes (CTL) response (55). Furthermore, it activates and expands T-helper cells, which induce B-cell growth and Ab production (55). After the second challenge, the complete protection of survived mice immunized by AAV1 tended to have better induction and maintenance of adaptive immune responses than AAV5. It means that not only humoral immune responses, but also other immune mechanism required for AAVs to induce long term protection. Our current study exhibited that a boosting of AAV1-Pf(s25-CSP) after initially prime with m8Δ-based vaccine could sustain sterile protection against three times repeated sporozoites challenges. Prior work documented that regulatory T cell (Tregs) and exhausted T cells were responsible for controlling a cytotoxic immune response led to persisting long-term transgene expression after AAV1 delivery (56). Therefore, it requires further investigation in future studies.

Regarding the use of these vaccination regimens as transmission-blocking vaccine (TBV), the high level of TB activity conferred by the m8Δ/AAV5-Pf(s25-CSP) or m8Δ/AAV1-Pf(s25-CSP) vaccination regimen is strongly correlated with the induction of high-level of anti-Pfs25 total IgG titer. As shown in previous studies, AAV1-based vaccine initially prime with either AdHu5 (26, 27) or m8Δ successfully elicited durable anti-Pfs25 with induced a high level TRA and TBA. Currently, TBV studies almost develop vaccine using Pfs25, a 25-kDa antigen that express on the surface of zygotes and ookinetes (57). The production of high level of anti-Pfs25 IgG achieved a significant transmission-blocking activity (10, 58, 59). In this study, our new vaccine regimen, m8Δ/AAV5-Pf(s25-CSP) achieved high expected outcome. The combination of PE and TB immunity of this regimen with high-level efficacies is a superiority over the PEV RTS, S/AS01 and TBV ChAd3/MVA Pfs25-IMX313 for a new-generation malaria vaccine (57). The m8Δ/AAV5-based vaccine may provide an alternative tool to the m8Δ/AAV1-based

for reducing mortality against malaria infection in endemic areas (12) and supporting malaria elimination program to meet the goals for 2030 set by WHO malaria vaccine roadmap (5).

In summary, this is the first report shows immunogenicity and protective efficacy of AAV5 vectors as a malaria booster vaccine regimen. AAV5-based malaria vaccine can induce robust Ab responses against two major malaria stage's antigens, achieves high-level protection rate, and provides strong TB activity as good as AAV1-based vaccine. Overall, AAV5-based malaria vaccine deserves further evaluation as an alternative vaccine vector if pre-existing anti-AAV1 antibody avoid vaccine re-administration with the same serotype and transgene expression.

ACKNOWLEDGMENT

I would like to express my most profound appreciation to Professor Shigeto Yoshida as my supervisor, who has continuously supported me throughout my study in his laboratory. Without his guidance and persistence help, this dissertation would not have been possible complete.

I also thank Professor Mitsuhiro Iyori, who has given some valuable input in our experiments to maintain our research's scientific quality and managed some of the works in this study that his expertise could only conduct.

I also thank Dr. Tetsushi Mizuno, who has been my great mentor in my first year of study. I am grateful to Yu-ichi Abe for his assistance during the protective efficacy study and Iroha Yamagoshi for the immunogenicity study. Many thanks to Mohammad Shahnawaj for his continuous technical support.

I also thank Dr. Yenni Yusuf, who has given chance and recommendation to pursue a Ph.D. study in Prof. Yoshida's laboratory and who has given me much support during my Ph.D. life in Kanazawa.

A big thank you to my father and mother, my father- and mother-in-law for their big motivation and support during my Ph.D. study during the problematic life in the SARS-CoV-2 pandemic era. Big love to my family, wife, and children for their patience for two years waiting to enter Japan. They had spent most of their time with other people, babysitters in Jakarta, and teachers in nursery school in Kanazawa when they finally arrived in Kanazawa at the end of my study.

I also thank Izumiya-san, who has helped manage some of our matters. I also thank all other lab members for the cooperation, good communication, and friendship that make the lab

environment more cheerful. Thank you to Dr. Yutaro Yamamoto, Dr. Akihiko Sakamoto, Prof. Hiroaki Mizukami, Dr. Andrew M. Blagborough, and Prof. Hisatoshi Shida, who have contributed to my study and publication.

Finally, my highest gratitude to my scholarship provider, the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan, who has spent lots of financial support for my study and living in Japan. Furthermore, to Dr. Anna Rozaliyani (Head of Parasitology Department of Faculty of Medicine Universitas Indonesia), Prof. Ari Fahrial Syam (Dean of Faculty of Medicine of Universitas Indonesia), and Dr. Rizalinda Sjahril (Head of Microbiology Department, Faculty of Medicine, Hasanuddin University), Prof. Budu (Dean of Faculty of Medicine Hasanuddin University) for their recommendation and support during MEXT scholarship application process and my Ph.D. study. In addition, to all my seniors, Dr. Firdaus Hamid, Dr. Fariz Nurwidya, Dr. Achmad Zaki, Dr. Eka Ginandjar, Dr. Fakhrur Razi and Dr. Ahmad Jamaluddin, for their motivation and support during my Ph.D. study.

REFERENCES

1. Ferguson NM. Challenges and opportunities in controlling mosquito-borne infections. Nature. 2018;559(7715):490-7.
2. World Health Organization. World malaria report 2021. Geneva: World Health Organization; 2021. Licence: CC BY-NC-SA 3.0 IGO. Geneva2021 [Available from: https://www.who.int/publications/i/item/9789240040496.
3. Remy V, Zollner Y, Heckmann U. Vaccination: the cornerstone of an efficient healthcare system. J Mark Access Health Policy. 2015;3.
4. Birkett AJ. Building an effective malaria vaccine pipeline to address global needs. Vaccine. 2015;33(52):7538-43.
5. Moorthy VS, Newman RD, Okwo-Bele JM. Malaria vaccine technology roadmap. Lancet. 2013;382(9906):1700-1.
6. World Health Organization. Full Evidence Report on the RTS,S/AS01 Malaria Vaccine. Geneva, Switzerland: Department of Immunization, Vaccines and Biologicals (IVB), WHO; 2021 October, 2021.
7. Laurens MB. RTS,S/AS01 vaccine (Mosquirix): an overview. Hum Vaccin Immunother. 2020;16(3):480-9.
8. Chichester JA, Green BJ, Jones RM, Shoji Y, Miura K, Long CA, et al. Safety and immunogenicity of a plant-produced Pfs25 virus-like particle as a transmission blocking vaccine against malaria: A Phase 1 dose-escalation study in healthy adults. Vaccine. 2018;36(39):5865-71.
9. Sagara I, Healy SA, Assadou MH, Gabriel EE, Kone M, Sissoko K, et al. Safety and immunogenicity of Pfs25H-EPA/Alhydrogel, a transmission-blocking vaccine against

Plasmodium falciparum: a randomised, double-blind, comparator-controlled, dose-escalation study in healthy Malian adults. Lancet Infect Dis. 2018;18(9):969-82.

10. Talaat KR, Ellis RD, Hurd J, Hentrich A, Gabriel E, Hynes NA, et al. Safety and Immunogenicity of Pfs25-EPA/Alhydrogel(R), a Transmission Blocking Vaccine against Plasmodium falciparum: An Open Label Study in Malaria Naive Adults. PLoS One. 2016;11(10):e0163144.
11. Wu Y, Ellis RD, Shaffer D, Fontes E, Malkin EM, Mahanty S, et al. Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51. PLoS One. 2008;3(7):e2636.
12. Blagborough AM, Churcher TS, Upton LM, Ghani AC, Gething PW, Sinden RE. Transmission-blocking interventions eliminate malaria from laboratory populations. Nat Commun. 2013;4:1812.
13. Miura K, Takashima E, Deng B, Tullo G, Diouf A, Moretz SE, et al. Functional comparison of Plasmodium falciparum transmission-blocking vaccine candidates by the standard membrane-feeding assay. Infect Immun. 2013;81(12):4377-82.
14. Skinner J, Huang CY, Waisberg M, Felgner PL, Doumbo OK, Ongoiba A, et al. Plasmodium falciparum Gametocyte-Specific Antibody Profiling Reveals Boosting through Natural Infection and Identifies Potential Markers of Gametocyte Exposure. Infect Immun. 2015;83(11):4229-36.
15. Bliss CM, Drammeh A, Bowyer G, Sanou GS, Jagne YJ, Ouedraogo O, et al. Viral Vector Malaria Vaccines Induce High-Level T Cell and Antibody Responses in West African Children and Infants. Mol Ther. 2017;25(2):547-59.
16. Ewer KJ, Sierra-Davidson K, Salman AM, Illingworth JJ, Draper SJ, Biswas S, et al. Progress with viral vectored malaria vaccines: A multi-stage approach involving "unnatural immunity". Vaccine. 2015;33(52):7444-51.

17. Prasad KM, Xu Y, Yang Z, Acton ST, French BA. Robust cardiomyocyte-specific gene expression following systemic injection of AAV: in vivo gene delivery follows a Poisson distribution. Gene Ther. 2011;18(1):43-52.
18. Wang D, Tai PWL, Gao G. Adeno-associated virus vector as a platform for gene therapy delivery. Nat Rev Drug Discov. 2019;18(5):358-78.
19. Zincarelli C, Soltys S, Rengo G, Rabinowitz JE. Analysis of AAV serotypes 1-9 mediated gene expression and tropism in mice after systemic injection. Mol Ther. 2008;16(6):1073-80.
20. Heilbronn R, Weger S. Viral vectors for gene transfer: current status of gene therapeutics. Handb Exp Pharmacol. 2010(197):143-70.
21. Naso MF, Tomkowicz B, Perry WL, 3rd, Strohl WR. Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. BioDrugs. 2017;31(4):317-34.
22. Gao G, Alvira MR, Somanathan S, Lu Y, Vandenberghe LH, Rux JJ, et al. Adeno-associated viruses undergo substantial evolution in primates during natural infections. Proc Natl Acad Sci U S A. 2003;100(10):6081-6.
23. Gao G, Vandenberghe LH, Alvira MR, Lu Y, Calcedo R, Zhou X, et al. Clades of Adeno-associated viruses are widely disseminated in human tissues. J Virol. 2004;78(12):6381-8.
24. Lorain S, Gross DA, Goyenvalle A, Danos O, Davoust J, Garcia L. Transient immunomodulation allows repeated injections of AAV1 and correction of muscular dystrophy in multiple muscles. Mol Ther. 2008;16(3):541-7.
25. Xiao W, Chirmule N, Berta SC, McCullough B, Gao G, Wilson JM. Gene therapy vectors based on adeno-associated virus type 1. J Virol. 1999;73(5):3994-4003.
26. Yusuf Y, Yoshii T, Iyori M, Yoshida K, Mizukami H, Fukumoto S, et al. Adeno-Associated Virus as an Effective Malaria Booster Vaccine Following Adenovirus Priming. Front Immunol. 2019;10:730.

27. Yusuf Y, Yoshii T, Iyori M, Mizukami H, Fukumoto S, Yamamoto DS, et al. A Viral-Vectored Multi-Stage Malaria Vaccine Regimen With Protective and Transmission-Blocking Efficacies. Front Immunol. 2019;10:2412.
28. Kenner J, Cameron F, Empig C, Jobes DV, Gurwith M. LC16m8: an attenuated smallpox vaccine. Vaccine. 2006;24(47-48):7009-22.
29. Kidokoro M, Shida H. Vaccinia Virus LC16m8 as a Vaccine Vector for Clinical Applications. Vaccines (Basel). 2014;2(4):755-71.
30. Mimuro J, Mizukami H, Shima M, Matsushita T, Taki M, Muto S, et al. The prevalence of neutralizing antibodies against adeno-associated virus capsids is reduced in young Japanese individuals. J Med Virol. 2014;86(11):1990-7.
31. Khatri A, Shelke R, Guan S, Somanathan S. Higher Seroprevalence of Anti-Adeno-Associated Viral Vector Neutralizing Antibodies Among Racial Minorities in the United States. Hum Gene Ther. 2022;33(7-8):442-50.
32. Boutin S, Monteilhet V, Veron P, Leborgne C, Benveniste O, Montus MF, et al. Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. Hum Gene Ther. 2010;21(6):704-12.
33. Stanford S, Pink R, Creagh D, Clark A, Lowe G, Curry N, et al. Adenovirus-associated antibodies in UK cohort of hemophilia patients: A seroprevalence study of the presence of adenovirus-associated virus vector-serotypes AAV5 and AAV8 neutralizing activity and antibodies in patients with hemophilia A. Res Pract Thromb Haemost. 2019;3(2):261-7.
34. Majowicz A, Nijmeijer B, Lampen MH, Spronck L, de Haan M, Petry H, et al. Therapeutic hFIX Activity Achieved after Single AAV5-hFIX Treatment in Hemophilia B Patients and NHPs with Pre-existing Anti-AAV5 NABs. Mol Ther Methods Clin Dev. 2019;14:27-36.

35. Miesbach W, Meijer K, Coppens M, Kampmann P, Klamroth R, Schutgens R, et al. Gene therapy with adeno-associated virus vector 5-human factor IX in adults with hemophilia B. Blood. 2018;131(9):1022-31.
36. Nathwani AC, Reiss UM, Tuddenham EG, Rosales C, Chowdary P, McIntosh J, et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. N Engl J Med. 2014;371(21):1994-2004.
37. Zabner J, Seiler M, Walters R, Kotin RM, Fulgeras W, Davidson BL, et al. Adeno-associated virus type 5 (AAV5) but not AAV2 binds to the apical surfaces of airway epithelia and facilitates gene transfer. J Virol. 2000;74(8):3852-8.
38. Hildinger M, Auricchio A, Gao G, Wang L, Chirmule N, Wilson JM. Hybrid vectors based on adeno-associated virus serotypes 2 and 5 for muscle-directed gene transfer. J Virol. 2001;75(13):6199-203.
39. Paneda A, Vanrell L, Mauleon I, Crettaz JS, Berraondo P, Timmermans EJ, et al. Effect of adeno-associated virus serotype and genomic structure on liver transduction and biodistribution in mice of both genders. Hum Gene Ther. 2009;20(8):908-17.
40. Nieto K, Stahl-Hennig C, Leuchs B, Muller M, Gissmann L, Kleinschmidt JA. Intranasal vaccination with AAV5 and 9 vectors against human papillomavirus type 16 in rhesus macaques. Hum Gene Ther. 2012;23(7):733-41.
41. Steel JC, Di Pasquale G, Ramlogan CA, Patel V, Chiorini JA, Morris JC. Oral vaccination with adeno-associated virus vectors expressing the Neu oncogene inhibits the growth of murine breast cancer. Mol Ther. 2013;21(3):680-7.
42. Goodman AL, Blagborough AM, Biswas S, Wu Y, Hill AV, Sinden RE, et al. A viral vectored prime-boost immunization regime targeting the malaria Pfs25 antigen induces transmission-blocking activity. PLoS One. 2011;6(12):e29428.

43. Iyori M, Blagborough AM, Sala KA, Nishiura H, Takagi K, Yoshida S. Protective efficacy of an IL-12-expressing baculoviral malaria vaccine. Parasite Immunol. 2017;39(12).
44. Xin KQ, Mizukami H, Urabe M, Toda Y, Shinoda K, Yoshida A, et al. Induction of robust immune responses against human immunodeficiency virus is supported by the inherent tropism of adeno-associated virus type 5 for dendritic cells. J Virol. 2006;80(24):11899-910.
45. Yoshida K, Iyori M, Blagborough AM, Salman AM, Dulal P, Sala KA, et al. Adenovirus-prime and baculovirus-boost heterologous immunization achieves sterile protection against malaria sporozoite challenge in a murine model. Sci Rep. 2018;8(1):3896.
46. Yamamoto DS, Yokomine T, Sumitani M, Yagi K, Matsuoka H, Yoshida S. Visualization and live imaging analysis of a mosquito saliva protein in host animal skin using a transgenic mosquito with a secreted luciferase reporter system. Insect Mol Biol. 2013;22(6):685-93.
47. Iyori M, Yamamoto DS, Sakaguchi M, Mizutani M, Ogata S, Nishiura H, et al. DAF-shielded baculovirus-vectored vaccine enhances protection against malaria sporozoite challenge in mice. Malar J. 2017;16(1):390.
48. Emran TB, Iyori M, Ono Y, Amelia F, Yusuf Y, Islam A, et al. Baculovirus-Induced Fast-Acting Innate Immunity Kills Liver-Stage Plasmodium. J Immunol. 2018;201(8):2441-51.
49. Epstein JE, Tewari K, Lyke KE, Sim BK, Billingsley PF, Laurens MB, et al. Live attenuated malaria vaccine designed to protect through hepatic CD8(+) T cell immunity. Science. 2011;334(6055):475-80.
50. Miura K, Swihart BJ, Deng B, Zhou L, Pham TP, Diouf A, et al. Transmission-blocking activity is determined by transmission-reducing activity and number of control oocysts in Plasmodium falciparum standard membrane-feeding assay. Vaccine. 2016;34(35):4145-51.

51. Louboutin JP, Wang L, Wilson JM. Gene transfer into skeletal muscle using novel AAV serotypes. J Gene Med. 2005;7(4):442-51.
52. Blagborough AM, Delves MJ, Ramakrishnan C, Lal K, Butcher G, Sinden RE. Assessing transmission blockade in Plasmodium spp. Methods Mol Biol. 2013;923:577-600.
53. Shahnaij M, Iyori M, Mizukami H, Kajino M, Yamagoshi I, Syafira I, et al. Liver-Directed AAV8 Booster Vaccine Expressing Plasmodium falciparum Antigen Following Adenovirus Vaccine Priming Elicits Sterile Protection in a Murine Model. Front Immunol. 2021;12:612910.
54. Klamroth R, Hayes G, Andreeva T, Gregg K, Suzuki T, Mitha IH, et al. Global Seroprevalence of Pre-existing Immunity Against AAV5 and Other AAV Serotypes in People with Hemophilia A. Hum Gene Ther. 2022;33(7-8):432-41.
55. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392(6673):245-52.
56. Gernoux G, Gruntman AM, Blackwood M, Zieger M, Flotte TR, Mueller C. Muscle-Directed Delivery of an AAV1 Vector Leads to Capsid-Specific T Cell Exhaustion in Nonhuman Primates and Humans. Mol Ther. 2020;28(3):747-57.
57. de Graaf H, Payne RO, Taylor I, Miura K, Long CA, Elias SC, et al. Safety and Immunogenicity of ChAd63/MVA Pfs25-IMX313 in a Phase I First-in-Human Trial. Front Immunol. 2021;12:694759.
58. Li Y, Leneghan DB, Miura K, Nikolaeva D, Brian IJ, Dicks MD, et al. Enhancing immunogenicity and transmission-blocking activity of malaria vaccines by fusing Pfs25 to IMX313 multimerization technology. Sci Rep. 2016;6:18848.
59. Kubler-Kielb J, Majadly F, Wu Y, Narum DL, Guo C, Miller LH, et al. Long-lasting and transmission-blocking activity of antibodies to Plasmodium falciparum elicited in mice by protein conjugates of Pfs25. Proc Natl Acad Sci U S A. 2007;104(1):293-8.