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Original Article

Safety, immunogenicity, and cross-species protection of a plasmid DNA encoding *Plasmodium falciparum* SERA5 polypeptide, microbial epitopes and chemokine genes in mice and olive baboons

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Abstract

Incorporation of biomolecular epitopes to malarial antigens should be explored in the development of strain-transcending malarial vaccines. The present study sought to determine safety, immunogenicity and cross-species efficacy of *Plasmodium falciparum* serine repeat antigen 5 polypeptide co-expressed with epitopes of Bacille-Calmette Guerin (BCG), tetanus toxoid (TT) and a chemokine gene. Olive baboons and BALB/c mice were randomly assigned into vaccine and control groups. The vaccine group animals were primed and boosted twice with pIRES plasmids encoding the SERA5 + BCG + TT alone, or with either CCL5 or CCL20 and the control group with pIRES plasmid vector backbone. Mice and baboons were challenged with *P. berghei* ANKA and *P. knowlesi* H strain parasites, respectively. Safety was determined by observing for injection sites reactogenicities, hematology and clinical chemistry. Parasitaemia and survivorship profiles were used to determine cross-species efficacy, and T cell phenotypes, Th1-, Th2-type, T-regulatory immune responses and antibody responses were assessed to determine vaccine immunogenicity. The pSeBCGTT plasmid DNA vaccines were safe and induced Th1-, Th2-type, and T-regulatory responses vaccinated animals showed enhanced $CD4^+$ ($P < 0.01$), CD 8^+ T cells ($P < 0.001$) activation and IgG anti-SE36 antibodies responses ($P < 0.001$) at week 4 and 8 post vaccination compared to the control group. Vaccinated mice had a 31.45-68.69% cumulative parasite load reduction and 60% suppression in baboons ($P < 0.05$) and enhanced survivorship ($P < 0.001$) with no clinical signs of malaria compared to the control group. The results showed that the vaccines were safe, immunogenic and conferred partial cross-species protection.

Keywords: malaria, DNA vaccines, serine repeat antigen, chemokines, cross-species, protection, immunogenicity, safety

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Introduction

The rapid emergence of malaria parasite drug resistance to drugs of choice and anopheline mosquito vector insecticide resistance threatens current gains made in rolling back malaria[1]. This has reinvigorated the search for effective human malaria vaccines[2]. There is mounting concern about emerging infections with the zoonotic simian malaria parasite, *Plasmodium knowlesi,* which is not only severe and fatal but also on the rise in people living in forested areas of South East Asia[3–7]. Furthermore, the prevalence of infections with the less prevalent *P. ovale* and *P. malariae* species seem to be increasing despite reported decreasing morbidity and mortality rates caused by *P. falciparum* worldwide[1,8]. This alarming trend suggests that future malaria vaccine development efforts should include the development of DNA based vaccines that are safe, immunogenic, and can confer cross-species protection. However, the current progress toward malaria vaccines development has been slow largely due to malaria parasite antigenic diversity and allelic heterogeneity, gaps in our knowledge about protective antigens, appropriate adjuvants for human use, and lack of suitable animal models for pre-clinical vaccine evaluation[9–10].

Vaccine development efforts aimed at circumventing problems associated with zoonotic malaria infections should focus on conserved malarial antigens such as *P. falciparum* SERA5. Such vaccines may offer an advantage of conferring cross-species protection against infections due to simian *P. knowlesi* and other human malaria parasite species which have not been targeted for vaccine development. Studies on population genetics have established that *Plasmodium* SERA genes in different species share sequences that are similar with each other during transcription and/or translation in the different family members[11–12]. The SERA5 antigen accumulates in the parasitophorous vacuole of late trophozoite and schizont stage, appears on the merozoite surface, and it is highly conserved among field and clinical isolates of *P. falciparum*[12]. Human clinical trials have established that SERA5 derived BK-SE36 candidate vaccine is not only safe and immunogenic but protects children against malaria attacks over a year of follow up[13]. Field studies have shown that high titers of IgG anti-SE36 antibodies are associated with protection against malaria in Ugandan children, adults and against placental malaria and low birth weights in pregnant women[14–15].

Malaria DNA based vaccines offer a more reliable, affordable and sustainable solution for disease control compared to conventional vaccines with respect to stability, manufacturing, storage, distribution, and ease of administration[16]. This is relevant in resource-poor settings with fragile public health infrastructures often lacking cold chains[17]. Multiple plasmids can be combined for elaboration of multivalent vaccines[18]. In non-human primates and humans, the efficacy of DNA vaccines is hindered by low immunogenicity due to lack of known pathogen-associated molecular patterns (PAMP) similar to those found in attenuated viral or bacterial vaccines to stimulate dendritic cells maturation and initiate strong adaptive immune responses that can confer long lasting protection[19]. However, the immunogenicity of DNA vaccines can be potentially increased by incorporation of immunomodulatory chemokines such as CCL5 and CCL20[20–22] as well as Bacille-Calmette Guerin (BCG) and tetanus toxoid (TT) epitopes. The C-C chemokines have been documented to possess adjuvant activity[23], and are readily expressed in primate and murine systems where they drive inflammatory responses during infections. In the present study, C-C chemotactic chemokines, CCL5 and CCL20 were chosen in the construction of plasmid DNA vaccines due to their capability to orchestrate dendritic and memory cell responses as well as recruitment and activation of T cells that mediate Th1/Th2-type and regulatory immune responses that confer protective blood stage immunity.

The olive baboon *(Papio anubis)* was utilized in the present study because it is a suitable experimental model for investigations of human vaccines; it is not only similar phylogenetically to humans but mounts similar immunological, clinical and physiologic responses[24]. We have previously demonstrated that the olive baboon is susceptible to experimental infection with *P. knowlesi*[25–27] and can be used in preclinical trials of *P. falciparum* vaccines[28]. The *P. knowlesi* H parasite strain used in the present study is a zoonotic malaria parasite that causes life-threatening disease if untreated in humans[4,29–30]. The baboon/*P. knowlesi* experimental model system has been developed for pre-clinical development of drugs[27] and understanding parasite biology in placental malaria[31–33]. Therefore, the present study investigated safety, immunogenicity and cross species efficacy of plasmid pSeBCGTT encoding a polypeptide of *P. falciparum* SERA5, Bacille-Calmette Guerin (BCG) and tetanus toxoid (TT) epitopes, and either CCL5 or CCL20 chemokine as immunomodulatory boosters in olive baboons. As a strategy, BALB/c mice were first used to provide preliminary data on immunogenicity and cross-species efficacy of pSeBCGTT DNA vaccines before pre-clinical evaluation in the olive baboon.

Materials and methods

Ethical statement

All experimental protocols for this study were reviewed and approved by the nationally accredited Institutional Review committee (IRC) of Institute of Primate Research (IPR) (Karen, Kenya) wide protocol number IRC/03/11. The experiments described herein are in accordance with ARRIVE guidelines[34]. Animals were housed and handled according to international guidelines on care and use of laboratory animals for biomedical use.

Parasites

Plasmodium knowlesi H strain blood stage parasite was retrieved from liquid nitrogen and propagated overnight in *in vitro* culture systems as described by Butcher[35]. The original parasite inoculum was Pk1. The (A +) clone was previously cloned by micro-manipulation and passaged in rhesus monkeys[36]. An inoculating dose of 1×10^5 *P. knowlesi* parasite infected red blood cells was used for baboon infection. The *P. berghei* ANKA strain parasites used were obtained from Kenya Medical Research Institute (KEMRI) and sequentially passaged in donor mice before being used for infecting experimental mice.

Animal models for *in vivo* analysis

One hundred twenty (120) BALB/c mice aged between six to eight weeks old and 9 male malaria naïve *Papio anubis* baboons (body weight ranging between 8 to 18 kg) were used in the study. Male baboons were used in the study due to availability and sex is not a risk for infection outcomes[25–27]. The animals were sourced and maintained at Institute of Primate Research (IPR) animal facility throughout the experimental period. The mice were maintained in ventilated cages and provided with heat sterilized food and water was *ad libitum*. Before use in the study, the olive baboons were screened for gut helminths, hemoprotozoan parasites, and mycobacterial infections according to institutional screening protocols and procedures.

Vaccine construct formulations

We chose a linear sequence of 38 amino acids from a region of *P. falciparum* SERA5 with confirmed protective activity[37] plus epitopes of BCG and TT (underlined) separated by glycine-proline GPGPG spacers (italicized) as the simple backbone of the vaccine because of their ability to mediate innate immunity in animal models. The proximal and distal underlined microbial sequences are epitopes of BCG and TT, respectively.

5'-*GPGPG*QVHFQPLPPAVVKL*GPGPG*ASQPGS-SEPSNPVSSGHSVSTVSVSQTSTSSEKQD-TIQ*GPGPG*QFIKANSKFIGITE-3'

The vaccine constructs consisted of synthetic DNA corresponding to the above sequence which was codon-optimized for expression in mammalian cells and sub-cloned into a cloning site of the mammalian expression vector pIRES by GeneArt (Regensburg, Germany). The resulting plasmid was designated as pSeBCGTT. A second cloning event resulted in pSeBCGTT encoding either CCL5 or CCL20 in the other cloning site, pSeBCGTT (expressing malaria and microbial epitopes), pSeBCGTT with CCL5, pSeBCGTT with CCL20, and pIRES alone without insert were dissolved in Tris EDTA buffer (TE pH 7.2) at a concentration of 1 mg/mL.

Experimental design

Mice were randomly assigned into pSeBCGTT (n = 24), pSeBCGTT /CCL5 (n = 24), pSeBCGTT/CCL20 (n = 24), pIRES (n = 24) and naïve (n = 24) groups. The vaccine groups received three doses of the vaccine at three-week intervals (days 0, 21 and 42) into each anterior *Musculus tibialis* (100 μg per muscle for total 200 μg DNA per dose) in TE buffer (pH 7.2). Three weeks after the last vaccination, all groups of mice were challenged by intra-peritoneal injection with 1×10^5 *P. berghei* ANKA infected red blood cells. Data on appetite, demeanour, and adverse reactions at the injection site was collected. Parasitaemia and parasite kinetic profiles were determined by microscopic examination of Giemsa-stained thin blood smears. The survival rates of infected mice were determined as a percentage of the mice surviving per group after challenge infection.

Olive baboons were randomly assigned into pSeBCGTT/CCL5 (n = 3), and pSeBCGTT/CCL20 (n = 3) vaccinated groups that received intramuscularly three doses of 333.3 μg/mL at four week intervals; and the control group (n = 3) similarly received pIRES in Tris EDTA buffer (pH 7.2). Data collected from non-vaccinated baboons was used to represent the naïve responses before commencement of vaccinations. After 1, 3, 7, and 14 days post priming, injection sites were examined for localized adverse reactions. The parameters checked for included induration, erythema, skin swelling, warmth, ulceration and regional lymphadenopathy by a veterinarian unaware of group assignments.

Venous peripheral blood was obtained for peripheral blood mononuclear cells (PBMC) isolation, complete blood count (CBC), serum separation and clinical chemistry. Four weeks after the last vaccination all monkeys were sedated and intravenously inoculated with the challenge dose of 1×10^5 *P. knowlesi* parasite infected red blood cells. Parasitaemia and parasite kinetic profiles were monitored daily by microscopic enumeration of the number of parasitized red blood cells per μL of baboon blood.

Clinical chemistry and hematology

To determine vaccine safety and tolerability, venous EDTA blood was collected every four weeks prior to vaccinations from all baboons after sedation with a mixture of 10 mg/kg Ketamine HCL and 0.5 mg/kg xylazine (Agrar, Holland). Haematological indices were analyzed using Beckman Coulter ACT 5 Diff. CP (USA). The analyses included complete blood cell counts, hematocrit, hemoglobin, mean corpuscular volume, platelet counts, erythrocyte counts and leukocyte counts. Sera were also collected for determination of clinical chemistry parameters using Humalyzer 2000 (Human, Germany) machine. The concentrations of creatinine phosphokinase (CK-MB), total bilirubin, direct bilirubin, alanine aminotransferase (ALAT/GPT), aspartate aminotransferase (ASAT/GOT), urea, and total protein concentrations were measured according to the manufacturer's instructions.

***In vitro* cell culture and analyses**

Isolation and preservation of PBMC

Venous whole blood was collected in Alsevers' solution (pH 7.2) from all baboons prior to vaccination and used as a source of PBMC. PBMC were isolated from peripheral blood by density gradient centrifugation using lymphocyte separation medium (Organon Teknika, Durham, NC) at 800 g for 25 minutes. The separated cells were washed twice in RPMI 1640 (GIBCO, NY) at 800 g for 10 minutes and re-suspended in RPMI 1640 containing 10% foetal bovine serum (GIBCO, NY), gentamycin and HEPES buffer (Sigma, St. Louis, MO). The cells were enumerated and viability determined by trypan blue dye exclusion method and adjusted into 1×10^7 cells/mL and either used immediately or cryopreserved for later use. To cryopreserve cells, aliquots of 1×10^7 cells/mL were re-suspended in 1 mL of RPMI 1640 containing 10% dimethyl sulfoxide (DMSO) and 25% heat inactivated fetal calf serum (FCSi), placed inside cryocell freezing containers filled with isopropanol, and kept at 4°C for 1 hour, overnight at 80°C and finally at -135°C until use.

Flow cytometric analysis

A 3-color flow cytometer (FACScan; BD, UK) was used to screen the monoclonal antibodies (mAb) for reactivity against baboon PBMCs. Briefly, prior to use, cryopreserved cells were rapidly thawed in a water bath at 37°C and immediately diluted 1:1 with RPMI 1640 medium containing 20% FCSi, 2 mmol/L L-glutamine, 100 μg/mL gentamycin followed by two washes in the same medium. The cells were adjusted to 1×10^6 cells/mL and dispensed into FACS tubes on ice and washed in PBS (pH7.2) containing 2% FCSi and 0.1% azide (FACS buffer). After centrifugation, the supernatant was aspirated, the cells mixed with 10 μL of either fluorescein-isothiocyanate (FITC)-conjugated mouse anti-human $CD3\varepsilon^+$ (BD, San Jose CA), peridin-chlorophyll (PerCP)-conjugated mouse anti-human $CD4^+$ (BD) and phycoerythrinin (PE)-conjugated mouse anti-human $CD8^+$ (BD) mAb. After staining and washing, the cells were fixed with 1% paraformaldehyde in PBS (pH 7.2) and fluorescence intensities and frequencies were measured. Cells were first analyzed by forward and side scatter and the lymphocyte population was gated to assess reactivity of the mAb with PBMCs. In each sample, 5,000 events in the lymphocyte gate were acquired and data analysis performed using Cellquest software (BD San Jose, CA).

Splenocyte isolation and proliferation

Mice were anaesthetized with 4% isoflurane inhalation and sacrificed to obtain spleens that were mashed in Petri dish with RPMI 1640. Splenic red blood cells were lysed using ammonium chloride lysis buffer (0.15 mol/L NH_4Cl, 1 mmol/L $KHCO_3$, 0.1 mmol/L EDTA). The cell suspension was washed twice with phosphate buffered saline (PBS) at 400 g for 5 minutes, re-suspended in RPMI 1640 (GIBCO, NY), and enumerated using trypan blue exclusion method under a light microscope. The isolated spleen cells were used for *in vitro* cellular proliferation and stimulation cultures where the cells were stimulated using recombinant SE-36. The unstimulated cells were used as controls. After 48 hours of incubation in a humidified 5% CO_2 incubator at 37 °C, cell supernatants were harvested and stored at -80°C until use.

Serological assays

IgG anti-SE36 antibody levels in vaccinated animals were determined by ELISA using recombinant SE36, expressed from SERA5[13]. An optimised SE36 antigen concentration of 4 μg/mL was used to coat 96 well Maxisorb ELISA plates (Nunc Rockilde, Denmark) overnight at 4 °C. Plates were washed, blocked with 1%

BSA in PBS Tween 20 for 1 hour at 37 °C, and test sera from animals added and incubated for 2 hours at 37 °C. Pre-immunization sera were used as negative controls. The plates were washed three times and rabbit anti-human IgG horseradish peroxidase (HRP) conjugated secondary antibody (Sigma) was added. After further incubation, the plates were washed as before and color development achieved by adding 3, 3′, 5, 5′-tetra-methyl-benzidene (TMB) substrate (KPL, Gaithersburg, MD). Samples were read at 630 nm using a Dynatech MRX microplate reader (Dynatech Laboratories, Sussex, UK) for 30 minutes. IgG anti-SE36 positive sera were defined as the serum samples with absorbance greater than the mean absorbance plus two standard deviations of negative (pre-immunization) control sera. Splenocyte culture supernatants were used to determine the concentrations of murine IFN-γ, TNF-α, IL-10 and IL-4 by cytokine-specific commercial ELISA kits according to the manufacturers' recommendations. TNF-α was determined using a mouse TNF-α ELISA kit (U-CyTech Biosciences, The Netherlands, CT303); IFN-γ ELISA used capture AN18 mAb and detection antibodies R4-6A2-biotin mAb (Mabtech, Sweden, Product Code: 3321-1A-6); IL-4 ELISA used capture 11B11 mAb and detection antibody BVD6-24G2-biotin mAb (Mabtech Product Code: 3311-1A-6) and IL-10 ELISA used capture mAb 2A5 and detection antibody mAb 16E3-biotin (Mabtech, Sweden, Product Code: 3431-1A-6). ρ-nitrophenyl-phosphate (pNPP) (Sigma) substrate was used. Samples and standards for each cytokine were read at 450 nm in a Dynatech MRX microplate reader (Dynatech Laboratories, Sussex, UK) and cytokine concentrations calculated using standard curves.

Statistical analysis

Statistical analysis was performed using GraphPad PRISM version 5.04 for Windows (Graph Pad Software, San Diego, CA). Results were described as mean ± standard error of the means and differences in mean (and standard errors) of continuous variables between the groups were compared using a two-way ANOVA test, followed by Bonferroni post-hoc analysis for multiple comparisons. Parasitaemia and survival rates were analyzed using Kaplan–Meier curves. A *P*-value of less than 0.05 was considered statistically significant.

Results

The pSeBCGTT plasmid DNA vaccines co-expressed with chemokines and microbial genes are safe in mice and olive baboons

To determine safety of the pSeBCGTT plasmid DNA vaccine formulations, injection sites were checked for adverse reactions. The results showed that the vaccine formulations had a clinically safe profile in mice and olive baboons because the injection sites did not show any adverse reactions related to vaccinations. Also there were no remarkable changes that were observed in the hematological and clinical biochemistry parameters among vaccine and control groups (***Table 1***).

The pSeBCGTT vaccine formulations demonstrated and enhanced activation and expansion of CD4 + and CD8 + T cell subsets, Ig G anti-SE36 antibodies in baboons and elevated Th1-/Th2-type and regulatory immune response in mice

To determine the plasmid DNA vaccine formulations

Table 1 **Haematological and clinical chemistry parameters**

Parameters	Normal values	pSeBCGTT/CCL5		pSeBCGTT/CCL20		pIRES plasmid	
		0	12	0	12	0	12
White blood cell count (10^3/μL)	6.7- 12.5	5.4±0.31	8.65±2.45	5.8±0.60	11.05±1.25	4.97±0.34	8.47±1.56
Haemoglobin (gm/dL)	11.7- 13.5	16.57±0.70	14±0.10	15.57±0.72	15.1±0.40	15.57±1.29	14.4±0.51
Platelets (10^3/μL)	150- 399	206.67±28.10	206.5±33.5	240±35.9	195.5±37.50	313.67±67.34	249±50.94
Lymphocyte count (%)	22- 50	35±0.58	37.5±0.70	43±6.351	56.65±15.95	62±9.24	55.6±0.35
Aspartate aminotransferase (U/L)	0- 40	27.57±4.30	26.65±3.55	31.43±2.22	49.35±0.45	29.57±1.17	31.87±3.62
Alanine aminotransferase (U/L)	0- 40	10.63±2.30	5.7±0.90	16.87±2.72	38.95±1.55	10.8±1.31	13±1.67
Creatinine (mg/dL)	0.5- 1.5	1±0.10	1±0.10	1.13±0.13	1.35±0.05	0.8±0.12	1.1±0.10
Creatinine Phosphokinase (U/L)	24- 200	109.43±59.63	145.5±77.5	136±2.65	215.5±15.50	83±29.68	74.47±25.59
Total bilirubin (mg/dL)	0- 0.4	0.4±0.00	0.5±0.10	0.5±0.25	0.25±0.21	0.37±0.20	0.37±0.20
Urea (mg/dL)	10- 50	43.67±2.58	48.25±2.55	28.93±3.62	37.05±2.55	38.8±3.07	38.2±1.69

Mean±SEM

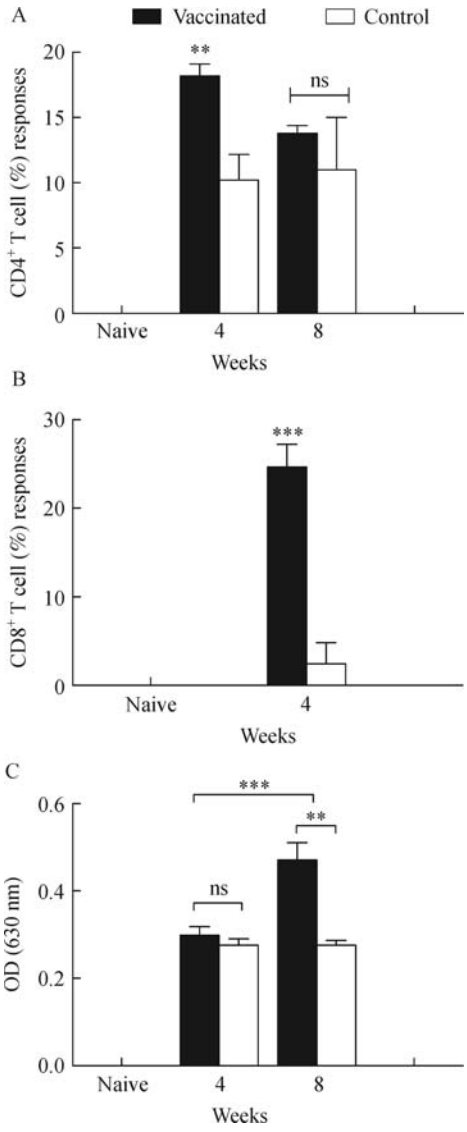

Fig. 1 **Immune responses of baboons during priming and 1st and 2nd boost immunizations.** (A) $CD4^+$ T cells, (B) $CD8^+$ T cells and (C) Ig G responses. The optical densities for each group are presented as means±standard error (SE) of antibody levels; levels of significance with * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

immunogenicity, the animals in the vaccine and control groups were sampled before and after vaccination and their humoral and cell-mediated immune responses determined. The antibody levels against SE-36 in baboons and cellular responses elicited by mice were determined by ELISA and flow cytometry was done to determine the expansion and activation of baboon T cell phenotypes. All baboons in the vaccine group had significantly higher (18%) activation of $CD4^+$ cells at week 4 than those of the control group (10%) ($P<0.01$) (***Fig. 1A***). After week 4 post vaccination, the vaccine group had higher $CD8^+$ T cell percent responses that were significantly elevated (24.6%) than those of the control group (2.41%) ($P<0.001$) (***Fig. 1B***).

The levels of serum IgG anti-SE36 antibody were increased in the vaccine group compared to the control group at week 8 post priming ($P<0.01$). The vaccine group had significantly high antibody titers of at week 8 compare to those of the control group ($P<0.001$) (***Fig. 1 C***).

After priming and two boosts, the levels of TNF-α in the pSeBCGTT/CCL20 vaccine group were significantly elevated at week 6 ($P<0.01$) and 9 ($P<0.05$) post-immunization compared to the other vaccine groups, the control group and the naïve group ($P<0.01$) (***Fig. 2A***). The levels of the pro-inflammatory cytokine, IFN-γ, were elevated at week 6 for the pSeBCGTT only group compared to the other vaccine groups, the naïve group and the control group ($P<0.001$) (***Fig. 2B***). After 3 and 6 weeks post-priming, the levels of regulatory cytokine, IL-10, were significantly elevated in the pSeBCGTT/CCL5 vaccine group ($P<0.01$) compared to the other vaccine groups, the naïve group and the control group (***Fig. 2C***). The levels of the Th2-type cytokine, IL-4, were comparable in the naïve group and the pSeBCGTT group 6 weeks after priming but later dropped in both groups at 9 weeks post-priming when they were elevated in the pSeBCGTT/CCL20 and pSeBCGTT/CCL5 groups ($P<0.001$) (***Fig. 2D***). Overall, the Th1- and Th2 type associated cytokines were markedly elevated in the plasmid DNA vaccinated groups compared to the naïve and control groups ($P<0.01$).

The pSeBCGTT plasmid DNA vaccine formulations co-expressing chemokines as immunological boosters showed partial cross protection against heterologous malaria challenge in baboons and mice

After primed and boost vaccinations both the mice and baboons were challenged with *Plasmodium* parasites and parasitaemia and survival rates were used to determine the plasmid DNA vaccine cross-species efficacy. Over the course of infection, mice vaccinated with pSeBCGTT/CCL5 ($P<0.001$, $r=0.988$) and pSeBCGTT/CCL20 ($P<0.05$, $r=0.758$) maintained lower parasitaemia compared to those vaccinated with pSeBCGTT only ($P<0.05$, $r=0.661$), pIRES ($P<0.01$, $r=0.964$) and naïve control group ($P<0.01$, $r=1.00$) (***Fig. 3A***). The naïve control group had the highest cumulative and peak number of parasites per μL of blood followed by the pSeBCGTT only group, pIRES, and pSeBCGTT/CCL20; pSeBCGTT/CCL5 had the lowest number of parasites. The mean cumulative and peak parasite burden in mice showed significant reduction of 31.45%–68.69% ($P<0.05$) and 34.58%–55.60% ($P>0.05$) (***Fig. 3 B*** and ***C***). Overall, the vaccine groups showed a better survival profile

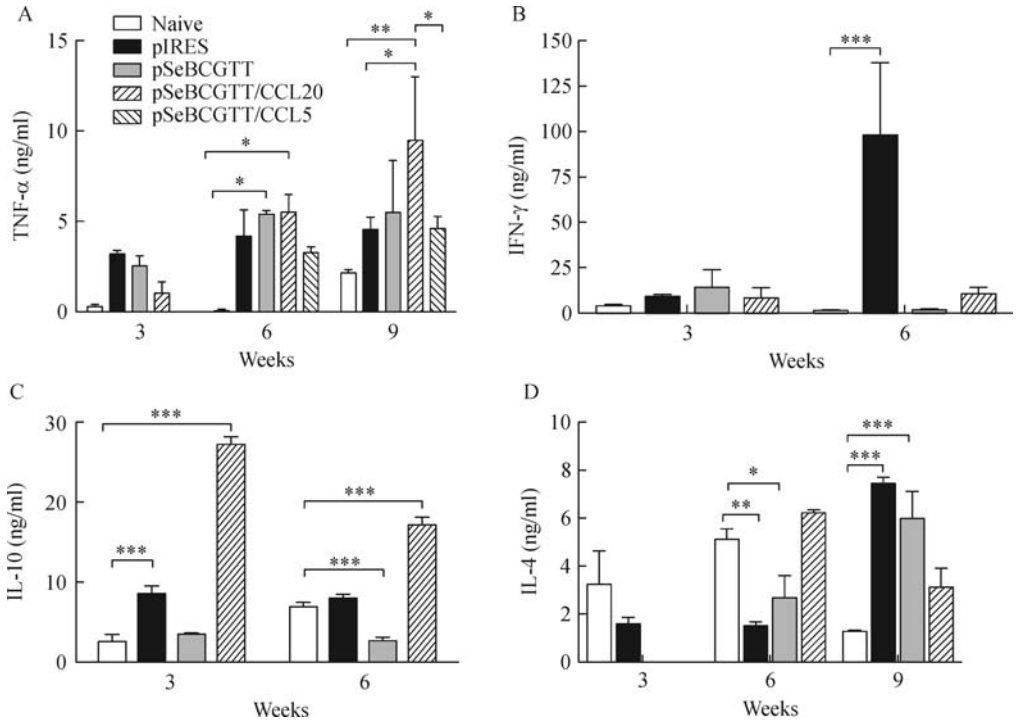

Fig. 2 **Th1/Th2 and T-regulatory immune responses (A) TNF-α, (B) IFN-γ, (C) IL-10 and (D) IL-4 in BALB/c mice vaccinated with various regimens at weeks 3, 6 and 9 post priming.** The cytokine concentrations for each group are presented as means±standard error (SE) (n = 10); levels of significance with * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

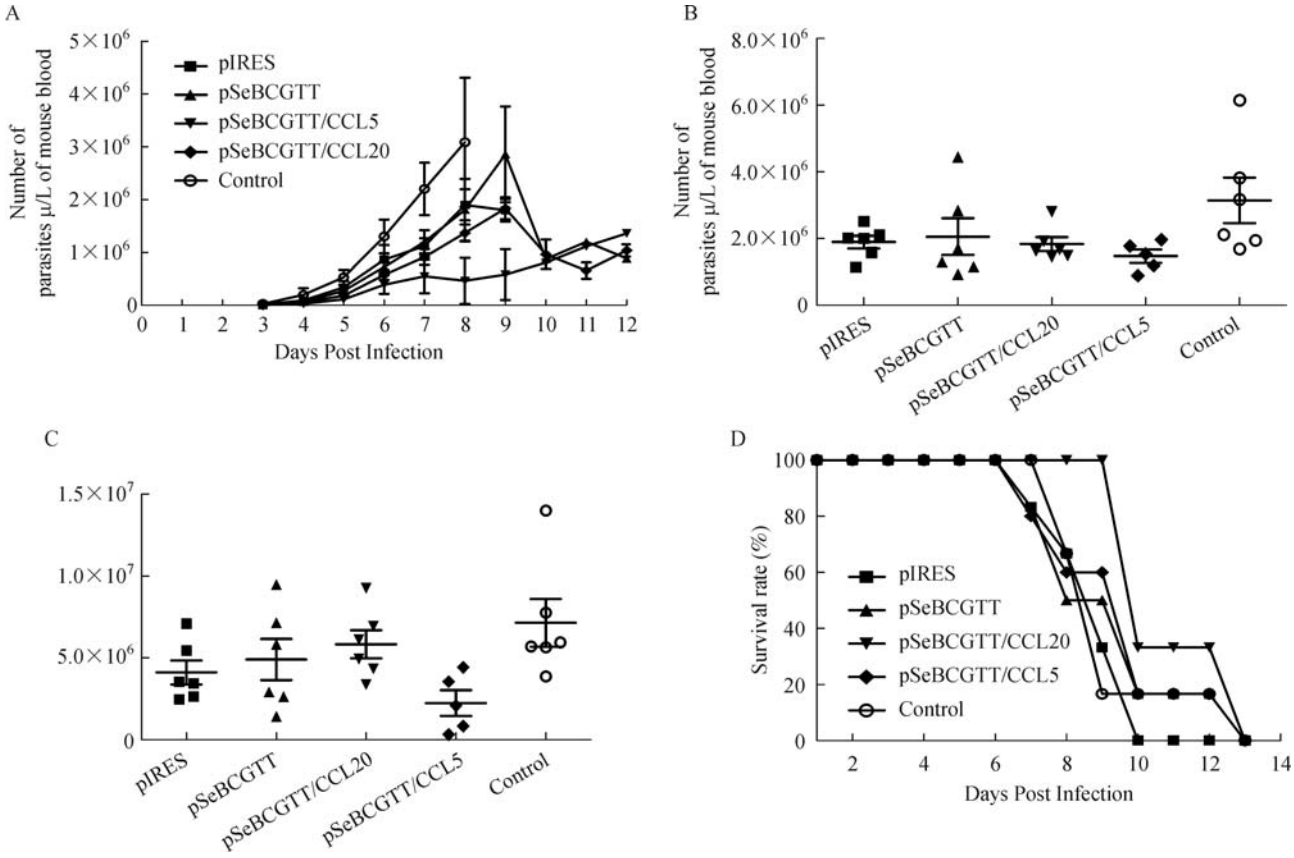

Fig. 3 **Cross-species efficacy of pSeBCGTT DNA vaccines against *P. berghei* ANKA challenge in mice.** (A) Parasite kinetics, (B) Peak parasitaemia, (C) Cumulative parasitaemia and (D) Mice survival rates. The number of parasites per μL of blood and survival rates for each group are presented as means±standard error (SE) (n = 10) of each group; levels of significance with * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

compared to the control group and the pIRES group ($P < 0.001$) (***Fig. 3D***).

As shown in ***Fig. 4***, the baboons in the vaccine groups sustained a higher number of parasites/μL of blood ($P > 0.05$, r = 0.357) compared to those in the control group ($P < 0.05$, r = 0.809). However, the baboons in the vaccine group showed a 60% decrease in mean cumulative parasitaemia. Furthermore, the vaccine group baboons survived a higher number of days with over 10,000 parasites/μL of blood. Despite this, they did not show clinical symptoms of malaria as to require antimalarial treatment (data not shown), compared to the control group that were treated with coartem™.

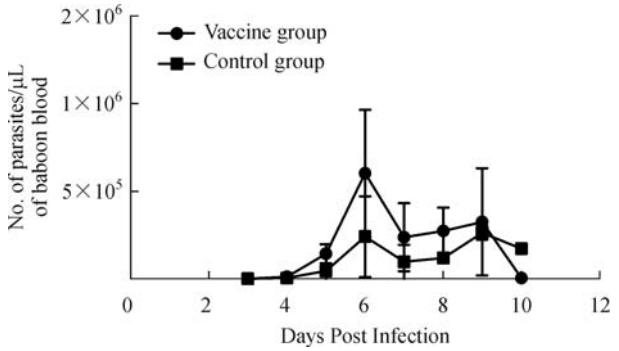

Fig. 4 **Parasitaemia profiles of Vaccine and control groups baboons challenged with *Plasmodium knowlesi* blood stage parasites.** The number of parasites per μL of blood and survival rates for each group are presented as means±standard error (SE); levels of significance with * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Discussion

In the present study, we successfully evaluated pre-clinical vaccine protocols in the olive baboon *(Papio anubis)*. Our findings showed that pSeBCGTT/chemokines plasmid DNA vaccine candidate were safe, immunogenic and conferred partial cross-species protection against *P. knowlesi* and *P. berghei* ANKA parasites in olive baboons and mice respectively. The findings verify that the olive baboon is a suitable model for vaccinations, monitoring clinical signs, multiple and sequential sample collection for hematology, clinical chemistry, immunological and parasitaemia analysis.

Our study has shown for the first time that the pSeBCGTT DNA vaccine candidate incorporating C-C chemokines is immunogenic in animal models and induces partial cross-species protection in mice against heterologous blood-stage *P. berghei* ANKA challenge and baboons against *P. knowlesi* H strain. Several studies have established that cell mediated immune responses are responsible for induction of protective immunity against blood stage malaria through their contribution in mediating antibody production[38]. The activation and elevation of the $CD4^+$ T cells responses in vaccinated group monkeys at week 4 indicates that the pSeBCGTT plasmid DNA vaccines with chemokines were able to mediate T cell activation and priming. This is further corroborated by the seropositivity to SE-36 antigen observed. Therefore, the findings suggest that the pSeBCGTT plasmid DNA vaccines may be influencing production of IgG antibodies that are specific to the SERA 5 sequences. Epidemiological studies have shown that the generation of malarial antigen specific antibodies is vital in the acquisition of immunity against malaria in people living in malaria-endemic areas[39]. Humans, mice and non-human primates acquire protection against malaria as a result of production of monoclonal antibodies against the surface molecules of malarial antigens in collaboration with effector T cells[40] resulting in acquisition of natural immunity after various episodes of malaria re-infections. Therefore, our findings suggest that the IgG anti-SE36 antibody elicited may be correlating to the partial cross-species protection against *P. knowlesi* observed in baboons. This is evident by the ability of vaccinated baboons to sustain a higher parasitaemia without clinical symptoms warranting antimalarial treatment compared to the control animals that were treated with three doses of coartem™. The enhanced antibody production observed in baboons may be attributed to either the co-expressions of C-C chemokines and microbial epitopes (BCG and TT) to the pSeBCGTT plasmid DNA vaccine or the two vaccine boosts done. The increase in counts of cytotoxic $CD8^+$ T cells phenotypes in the baboons shows evidence of effector cells activation being responsible for protective immunity against malaria parasites developing in the hepatocytes[41]. Our findings further showed that the pSeBCGTT vaccine co-expressed with CCL5 or CCL20 chemokines may have initiated antigen presentation to MHC class I molecules resulting in endogenous pathway activation of $CD8^+$ T cells[42]. This proofs the concept that the incorporation of immunomodulatory chemokines and microbial epitopes to plasmid DNA vaccines may have improved their immunogenicity.

The elevation levels of the pro-inflammatory cytokines, tumor necrosis factor (TNF)-α and interferon (IFN)-γ in the vaccine group mice observed may be associated with some levels cross-species protection conferment that was observed in the study. From other studies, it has been established that increase in the levels of IFN-γ with or without TNF-α is significantly associated with high levels of protection against malaria infection[43]. In the study, the pSeBCGTT plasmid DNA

vaccines in mice induced secretion of regulatory responses that may have influenced secretion of interleukin (IL)-4 cytokine, a signature cytokine for anti-inflammatory response. Therefore, suggest that the vaccines influenced the induction of Th1-, Th2-type and regulatory responses after priming and these responses were sustained as a result of subsequent boost vaccinations.

Development of a human malarial vaccine inducing simultaneous protection against multiple human and simian *Plasmodium* species will have enormous economic, safety, and production advantages. Here, we demonstrate for the first time the potential of pSeBCGTT incorporating C-C chemokines as well as microbial epitopes as a species transcending antigen inducing cross-species immunity and protection. The World Health Organization (WHO) launched the malaria vaccine technology road map in 2006. The road map has a landmark goal of a vaccine with 50% efficacy against severe disease and death to be developed by 2015[44]. This study shows that experimental vaccination with pSeBCGTT DNA in mice and baboons significantly suppressed parasitaemia up to about 60%. These results, supports for further evaluation of pSeBCGTT plasmid DNA vaccines with immunomodulatory chemokines as a multiple strain transcending malaria blood stage vaccine candidate.

Furthermore, the olive baboon model provides a useful platform for evaluation of malaria vaccines before phase 1 clinical trials. This study has further developed vaccine evaluation protocols allowing determination of vaccine safety, toxicity, tolerability, immunogenicity and cross protective efficacy of a plasmid DNA vaccine formulation. Due to phylogenetic closeness to humans baboons are increasingly becoming attractive for analysis of vaccine safety and immunogenicity[24], including use of chemokine adjuvants. Adjuvant safety and potency are important issues that frequently limit development of new formulations in malaria studies. The use of naked DNA approach with plasmids co-expressing single or multiple malaria antigens incorporating immunomodulatory chemokines are expected to gain more relevance in future malaria vaccine development efforts.

Single antigen malaria vaccines have shortcomings in protecting against *Plasmodium* in populations partly due to antigen allelic heterogeneity. Still a major challenge remains in the identification of potentially cross-protective antigens[45]. However, it is possible that novel protective antigens do exist in the malaria genome and need to be evaluated. The challenge in development of subunit vaccines for malaria and other infectious diseases has been the identification of safe and potent adjuvants that are capable of inducing effective cross-species protective immunity[9]. In support of this idea, developing pSeBCGTT plasmid DNA vaccine is relevant due to its highly conserved in nature 445 worldwide isolates of *P. falciparum.* This differs with other major vaccine malaria target antigen genes without strong evidence for positive selection as was detected using SNPs in the non-repeat regions of SERA[12]. The *Pf*SERA is a conserved merozoite surface antigen in various parasite species making it a potential choice for developing cross-protective malaria vaccine[11].

Notably, the polymorphic nature of *Pf*SERA5 does not appear to hamper the potency of the recombinant N-terminal domain (SE47') since antibodies raised against mouse, rat and monkey cross-reacted with several parasite lines[46–47]. Similarly, antibodies to AMA1 show variable levels of cross-inhibition of parasite growth *in vitro* when tested against heterologous strains of *P. falciparum*[48–50] suggesting that existing vaccine antigens, of which there are at least 15 blood-stage candidates[51–53] may be insufficient when delivered as single vaccines to protect against malaria populations in the field, due to allelic heterogeneity among antigens. Therefore, to be broadly protective, a malaria vaccine will need to circumvent human genetic diversity by use of highly conserved antigens such as *Pf*SERA.

A safe and feasible human malaria vaccine should protect humans against the multiplicity of strains circulating in endemic populations. This endeavor continues to encounter challenges attributable to scarcity of reliable and reproducible animal models for vaccine development[54]. Non-human primates such as olive baboons are well-suited as experimental models for vaccine development ahead of clinical evaluation because their systems are predictable of human. There is also need for novel and alternative adjuvants to modulate and enhance immune responses of DNA vaccines. Appropriate adjuvants for use in malaria vaccines are critically required and the olive baboon should be considered as an ideal platform for their development.

Novel concepts in malaria vaccine development must employ strategies that include as a major goal, the generation and maintenance of specific memory T cells. The use of chemokines as immune-booster is advantageous in attracting dendritic cells to the vaccination site, where they can take up antigen[55]. C-C chemokines such as CCL5, which enhance T helper 1 responses, and CCL20, which recruit and activate dendritic cells and memory T lymphocytes and have been incorporated into vaccine constructs[56–57]. New malaria vaccine

constructs incorporating CCL5 and CCL20 for boosting Th1- type immunity and sustaining immunological memory are required. This is further strengthened by the results our study. These two chemokines have potential to be used as immune-boosters in humans given their safety profile and effect on immune responses in addition to control of parasitaemia.

In conclusion, the plasmid pSeBCGTT encoding a polypeptide of *P. falciparum* SERA5, Bacille-Calmette Guerin (BCG) and tetanus toxoid (TT) epitopes, with either CCL5 or CCL20 chemokine as immune-boosters, has proven to be a strain-transcending conserved antigen. It has demonstrated potential for inclusion as part of a multi-antigen vaccine to synergise with other allelic heterogeneous candidates to provide broader coverage against multiple *Plasmodium* strains.

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