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Immunogenicity of recombinant BCG-based vaccine expressing the 22 kDa of serine repeat antigen (SE22) of *Plasmodium falciparum*

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Abstract. The *Plasmodium falciparum* serine repeat antigen (SERA) is one of the promising blood-stage malarial vaccine candidates. In this study, recombinant *Mycobacterium bovis* bacille Calmette-Guerin (rBCG) expressing the 22 kDa protein (SE22) from the 47 kDa N-terminal domain of serine repeat antigen (SERA), generated in favour of mycobacterium codon usage, elicited specific immune response in BALB/c mice with a mixed Th1/Th2 profile. Immunized sera containing high levels of specific IgG1 and IgG2a against the epitope (as determined by ELISA) were reactive with fixed *P. falciparum* merozoites as demonstrated by indirect immunofluorescence assay (IFA). Furthermore, the lymphocyte proliferative response to SE22 antigen from rBCG-immunized mice was higher than that of controls. The expression of intracellular cytokines (IL-2, IL-4 and IFNγ) in $CD4^+$- and $CD8^+$-cells was also enhanced following *in-vitro* stimulation with SE22. These findings indicate that a rBCG-based vaccine candidate expressing a blood-stage antigen of *P. falciparum* could enhance both humoral and cellular immune responses, thus paving the way for the rational use of rBCG as a vaccine candidate against malaria.

INTRODUCTION

A malarial vaccine is urgently needed because of the serious public health consequences of the disease in most tropical countries. Malaria is a problem in Malaysia especially in the rural areas of Sabah and Sarawak (Malaysia Health Ministry, 2004). An anti-malarial vaccine has long been a public priority. However, despite extensive research, a safe, effective and affordable anti-malarial vaccine remains elusive. Several candidate vaccines are however being developed, either at the pre-clinical stage (Kester *et al*., 2008; Genton *et al*., 2010) or have entered Phase I/II/III clinical trials (Barbosa *et al*., 2009; Agnandji *et al*., 2011; Asante *et al*., 2011; Leach *et al*., 2011; Lusingu *et al*., 2011).

The major symptoms of malaria are caused by the asexual blood-stage of the parasite life cycle and these forms of the parasites are an important target for the anti-malarial vaccine. The serine repeat antigen (SERA) of *Plasmodium falciparum* is an asexual blood-stage antigen produced in large amounts during the late trophozoite and schizont stages. SERA protein is secreted into the lumen of the parasitophorous vacuole via an endoplasmic reticulum (ER) - dependent pathway after removal of the 22 amino acid signal peptide (Delplace *et al*., 1987; Bzik *et al*., 1988; Fox & Bzik, 1994). SERA localizes at the parasitophorous vacuole as well as the surface of the developing merozoites (Perkin & Ziefer, 1994). Upon schizont rupture, SERA is processed into a 47 kDa N-terminal (P47), a 50 kDa central (P50), an 18 kDa C-terminal domain (P18) and a 6 kDa domain (P6).

SERA is a target of *in-vitro* parasite-inhibitory antibodies prior to or after

merozoites release. Previous studies have reported that the anti-SERA antibodies can agglutinate merozoite and schizonts and block merozoite dispersal (Perkin & Ziefer, 1994, Fox *et al*., 1997; Pang & Horii, 1998; Pang *et al*., 1999). The epitopes within the P47 domain provided significant protection to *Aotus* or squirrel monkeys from *P. falciparum* challenge (Inselburg *et al.*, 1991; 1993a and b; Fox *et al*., 1997). Okech *et al.*, (2001, 2006) reported a positive association between naturally induced antibody response to the 47 kDa domain and an increased protective immunity in adults in Uganda. This epidemiological study revealed that increased levels of IgG against the 47 kDa peptide correlated with lower parasitemia in the peripheral blood and the absence of fever in a group of children (Okech *et al.*, 2001, 2006).

A 22 kDa (SE22) conserved protein fragment from the N-terminal domain of SERA was studied (Puentes *et al*., 2000). As reported by Puentes *et al.* (2000), peptide encoding the YLKETNNAISFESNSGSLEKK found in the 47 kDa SERA has shown to have high erythrocyte binding activity. In addition, previous studies showed that antibodies produced against the amino acid with high erythrocyte binding activity might play important role in eliminating infection by agglutinating merozoites and block invasion (Pang *et al*., 1998, 1999).

Mycobacterium bovis bacille Calmette-Guerin (BCG) is used as a vaccine against human tuberculosis. The widespread use of BCG has demonstrated its safety and its potent immunogenicity. This has prompted its use as a vehicle or carrier for vaccines against other diseases. It is known as a strong inducer of a Th1-type response, and infection of live recombinant BCG expressing foreign antigens has been shown to elicit cell-mediated immunity directed toward the heterologous antigens (Leung *et al*., 2000; Nascimento *et al*., 2000; Mederle *et al*., 2002). In addition to inducing a cellular immune response, recombinant BCG can also induce significant levels of antibodies against foreign antigens (Mederle *et al*., 2002; Young *et al*., 2002; Rezende *et al*., 2005; Govan *et al*., 2006; Nurul & Norazmi, 2011). Therefore, this mycobacterium constitutes an ideal live vector for the presentation of foreign antigens to the immune system. Furthermore, BCG has been widely used as a vaccine against tuberculosis with an excellent safety record, can be administered at or any time after birth, and is substantially less expensive than most other vaccine formulations (Nascimento *et al*., 2000; Franco-Paredes *et al*., 2006).

In this study, we cloned and expressed SE22 of *P. falciparum* in BCG. The expression of the gene was driven by the heat shock protein 65 (*hsp65*) promoter of *Mycobacterium tuberculosis* and secretion of the antigen was modulated by the signal peptide from MPT63 of *M. tuberculosis*. The candidate vaccine was introduced into BALB/c mice and immunogenicity study was performed. The rBCG expressing SE22 induced humoral and cellular responses in immunized mice.

MATERIALS AND METHODS

Bacteria and media

The commercially available *Escherichia coli* TOP10 strain (Invitrogen, USA) was used in all initial cloning procedures. The recombinant *E. coli* TOP10 was grown in LB media supplemented with 50 µg/ml kanamycin (Sigma, USA). The *M. bovis* BCG Japan vaccine strain was cultured in 7H11 or 7H9 media (Difco Laboratories, USA) and rBCG was supplemented with 15 µg/ml kanamycin.

Synthesis and cloning of SE22

The 22 kDa N-terminal region of SERA of *P. falciparum* was designed based on the FCR3 strain; gene bank accession number: J03993, which comprised amino acid 91-248 (Table 1). In the present study, the SE22 was synthetically generated in favour of Mycobacterium codon usage by assembly PCR (Norazmi *et al*., 1999; Nurul *et al*., 2010). Briefly, 50 µl of PCR mixture was prepared containing 2.5 µM of synthetic oligonucleotides, 1.75 U Expand High Fidelity DNA polymerase (0.5 µl), 1 x MPCR buffer from High GC PCR* / MPCR* Optimization Kits (5 µl), 200 µM of each dNTPs, 2.5 µl

Table 1. Sequence of SE22 of *P. falciparum* FCR3 strain

D	T	I	Q	V	K	S	A	L	L
K	D	Y	M	G	L	K	V	T	G
P	C	N	E	N	F	I	M	F	L
V	P	H	I	Y	I	D	V	D	T
E	D	T	N	I	E	L	R	T	T
L	K	E	T	N	N	A	I	S	F
E	S	N	S	G	S	L	E	K	K
K	Y	V	K	L	P	S	N	G	T
T	T	G	E	Q	G	S	S	T	G
T	V	R	G	D	T	E	P	I	S
D	S	S	S	S	S	S	S	S	S
S	S	S	S	S	S	S	S	S	S
S	S	S	S	S	S	S	S	S	S
S	S	S	S	E	S	L	P	A	N
G	P	D	S	P	T	V	K	P	P
R	N	L	Q	N	I	C			

DMSO, 5 µl GC-Normalizer™ and nuclease free dH_2O. The mixture was amplified in a GeneAmp PCR System 2400 thermal cycler with an initial denaturation of 95°C for 60s followed by 55 cycles at 95°C for 60s, 55°C for 30s, 72°C for 30s and a final elongation time of 10 mins at 72°C.

A second PCR was performed on 5 µl of the amplicon from the first PCR using the following primers: sense 5' CATATGGACA CCATCCAGGTGAAG 3' and antisense 5' TGCTCT AGACTAGCAGATGTTCTGCAG 3' primers at a concentration of 2.5 µM each. The primers included the NdeI and XbaI restriction enzyme sites to facilitate cloning. The amplification of the SE22 was performed using the following PCR conditions: 40 cycles at 95°C for 60s, 65°C for 60s and 72°C for 30s. The 486 bp PCR product was cloned into the cloning vector pCR®2.1-TOPO® (Invitrogen) and confirmed by DNA sequencing.

The gene fragment encoding SE22 was re-cloned at the NdeI and XbaI sites of the shuttle vector, pNMN010 (previously constructed by our group), which is based on the pUC19 plasmid vector containing the hsp65 promoter from *M. tuberculosis*, the signal peptide MPT63 of *M. tuberculosis*, the mycobacterial origin of replication (oriM) and a kanamycin-resistant cassette. The resultant clone was confirmed by DNA sequencing and designated pNMN012. The plasmid was then transformed into BCG Japan strain by electroporation (Pulse Controller II & Gene Pulser® II, Bio-Rad, USA) with the pulse condition of 2.5 kV, 25 µF, and 600 Ω as previously described (Norazmi *et al.*, 1999). Recombinant clones were obtained via kanamycin selection and the recombinant BCG clone was designated rBCG012.

Expression of SE22 in BCG

The rBCG012 and BCG (negative control group) were grown in Middlebrook 7H9 broth containing 10% oleic acid/albumin/dextrose/catalase (OADC) and 0.5% Tween 80 and 15 µg/ml kanamycin at 37°C for 3 weeks. Following incubation, the rBCG cells were harvested by centrifugation at 4000 rpm for 10 mins. For expression analysis, the rBCG cells were lysed on ice by sonication (Ultrasonic Processor XL Sonicator) for 5 mins (15s on and 15s off). After sonication, the lysed cells were centrifuged at 14,000 rpm for 10 min and the pellet was discarded. The lysates were mixed with sample buffer (2% sodium dodecyl sulphate, SDS; 50 mM Tris) and subjected to 12% polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membrane (Hybond C, Amersham Biosciences UK Ltd.) at a constant current of 13V for 1 hour. Western blotting was carried out by first blocking the membrane with 5% skimmed milk in 0.05% Tween 20 in PBS (PBS-T20) for 1 hour. The membrane was washed 3x with PBS-T20 for 15 mins and reacted with the monoclonal

antibody, anti-SE47, which reacts with the N-terminus of SERA and includes the SE22 epitope (kind gift from Prof. Toshihiro Horii, Osaka University) for 1 hour at room temperature. Horseradish peroxidase-conjugated IgG antibodies (Dako, Japan) was used as a secondary antibody at a dilution of 1:1000 and the reaction was detected using the ECL™ Western Blotting Detection Kit according to the manufacturer's protocol (Amersham Biosciences, UK).

Preparation of rBCG vaccine candidate

The BCG and rBCG were cultured for 2 to 3 weeks in 7H9 broth containing 10% OADC and 0.5% Tween 80 supplemented with 15 µg/ml kanamycin (for rBCG). The number of cells used for vaccination was determined by calculating the colony forming unit (cfu). The method for calculating the cfu of BCG was based on the report by Zheng *et al.* (2002a). An $O.D_{600} \approx 0.1$ is equivalent to approximately $4x10^6$ cfu of BCG or rBCG. The cells were harvested by centrifugation at 4000 rpm for 10 mins. The supernatant was discarded and the cells were diluted to $2x10^6$ cfu per 200 µl of PBS+0.5% Tween 80 (PBS-T80) (Matsumoto *et al.*, 1998).

Animals and immunization protocol

Vaccination was performed intraperitoneally (i.p) with either 200 µl of $2x10^6$ cfu rBCG or BCG. Female BALB/c ($H\text{-}2^d$) mice (5/group) of 4-6 weeks old were boosted 3 weeks after primary injection and the final booster given after another 3 weeks. The animals were monitored after immunization to ensure that there were no adverse effects of the vaccine. Blood was collected from the tail vein before every immunization and just before sacrificing the mice. The collected blood was left to clot at 4°C overnight. The sera were obtained by centrifugation at 1500 rpm and stored at -20°C. All animal experiment studies were approved by the Animal Ethics Committee of Universiti Sains Malaysia.

Measurement of total IgG and IgG subclass antibodies by ELISA

SE22 purified recombinant protein that was produced in-house was diluted to 0.5 µg/ml with carbonate-bicarbonate coating buffer (Na_2CO_3-$NaHCO_3$). The diluted protein was then added to each well of a 96-well plate in a 100 µl volume and incubated overnight at 4°C. The plate was washed twice with PBS-T20 for 5 mins each and 200 µl of blocking buffer was added to each well and incubated at 37°C for 2 hours. The plate was then washed 5x (5 mins each) with PBS-T20 before the addition of 100 µl of mouse sera at a dilution of 1:1000. The plate was incubated for 2 hours at 37°C followed by 5 washing steps (5 mins each). One hundred µl of 1:2000 dilution of the appropriate secondary antibodies: either HRP conjugate goat anti-mouse IgG, rabbit anti-mouse IgG1, IgG2a and IgG2b or goat anti-mouse IgG3 was added to each well and incubated for 2 hours at 37°C. After washing 5x (5 mins each), 100 µl of 2,2'-azino-di(3-ethylbenzotiazolensulfonat) (ABTS®; Boehringer Mannheim, Germany) was added and the plate incubated for 30 mins. The reaction was stopped by the addition of 100 µl of 2N H_2SO_4 and the colour development was read at O.D 405 nm.

Indirect immunofluorescence assay (IFA)

The IFA was performed with sera obtained from immunized mice. For this assay, the slides which were coated with *P. falciparum* infected erythrocytes (kindly provided by Dr. Anthony A. Holder, National Institute for Medical Research, U.K) were fixed by immersing them in cold acetone at -20°C. Ten µl of serum was added to the slide and incubated for 30 mins at 37°C in a humid chamber. After incubation, the slides were washed with PBS+1% BSA (PBSA) and placed into a slide jar containing fresh PBSA. The slides were allowed to soak for approximately 5 mintues in the slide jar. A secondary antibody, anti-mouse IgG FITC diluted in 1:100 with fresh PBSA was applied onto the slide and incubated for another 30 mins at at 37°C in a humid chamber. The slides were washed as above and then dipped in a solution of 0.01% Evans Blue in PBSA. The slides were washed again and dried before mounting agent was added and coverslips placed on top. The slides were then visualized using a fluorescence microscope under 100x objective.

Analysis of intracellular cytokines

The splenocytes obtained from immunized mice were pooled and cultured at 2×10^6 cells/ml in 5 ml tissue culture tubes (BD Bioscienes, USA) in the presence of 5 µg/ml of SE22 protein and incubated at 37°C in 5% CO_2 for 72 h. Golgi/plug solution (10 µg/ml) (BD Bioscienes, USA) containing Brefeldin A was added to each tube 6 hours before harvesting. The cultures were washed twice with RPMI, resuspended in 50 µl staining buffer (PBS without Mg^{2+} or Ca^{2+}; 1% heat-inactivated FCS; 0.09% (w/v) sodium azide) and stained with 20 µl (0.05 mg/ml) anti-mouse $CD4^+$ or $CD8^+$ mAbs labeled with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) and anti-mouse $CD3^+$ mAb labeled with peridinin-chlorophyll protein (PerCP). The cells were incubated at 37°C in the dark for 30 min, washed with staining buffer and treated with 250 µl Cytofix/Cytoperm™ solution (BD Bioscienes, USA) containing paraformaldehyde and saponin for 20 min at 4°C. The cell pellet was washed twice and resuspended in 50 µl Perm/wash™ solution (BD Bioscienes, USA) containing saponin and sodium azide. The cells were then stained with 20 µl (0.05 mg/ml) anti-mouse IL-2 or IFN-γ (BD Bioscienes, USA) labeled with FITC or anti-mouse IL-4 conjugated with PE and incubated at 4°C for 30 min in the dark. Finally, the cells were washed twice with 1 ml Perm/wash™ solution, resuspended in 200 µl staining buffer and kept at 4°C prior to analyses in the FACSCalibur flow cytometer (BD Bioscienes, USA) using the CELLQuest Software (BD Bioscienes, USA).

RESULTS

Synthesis, cloning and expression of SE22 of *P. falciparum* in BCG

The synthetic gene coding for the 22 kDa N-terminal region of SERA of *P. falciparum*, FCR3 strain, amino acid 91-248 (accession number: J03993) which was driven by the hsp65 promoter of *M. tuberculosis* and secretion of the antigen was modulated by the signal peptide of MPT63 of *M. tuberculosis* was synthesized using assembly PCR technique with some modifications (Norazmi *et al*., 1999; Nurul *et al*., 2010). The sequence was designed using the OLIGO™ (National Bioscience) and MacDNAsis Ver.3.2 (Hitachi Software Engineering Co. Ltd) softwares. The synthetic SE22 was designed in favour of mycobacterium codon usage based on the frequently used codons in BCG (Dale & Patki, 1990) which is about 60% to 70% G+C compared to plasmodia genome which is about 82% A+T (Gardner *et al*., 2002).

Cell lysates of rBCG012 and the control cells, BCG were subjected to Western blotting analysis. Western blotting of rBCG012 using anti-SE47 showed that the SE22 gene was expressed in BCG with a band size 30 kDa (Fig. 1) corresponding to the expected molecular weight of SE22. No bands were observed by the BCG group.

Antibody response to SE22

Sera obtained from the immunized mice were tested by ELISA to identify the levels of total IgG and IgG subclasses against SE22. As shown in Fig. 2a, a significant level of anti-SE22-specific IgG antibody was observed in mice immunized with rBCG012 after the final boost ($p< 0.01$). Moreover, the level of total IgG was also significantly higher in mice immunized with rBCG012 compared to control groups.

Since the isotype of the antibody was considered important in determining the protective nature of the immune responses to malaria infection (Okech *et al*., 2001, 2006), the subtyping of IgG response in Balb/c mice induced upon rBCG012 immunization was carried out. Importantly, IgG subclass responses were found higher in mice immunized with rBCG012 compared to control groups. There was a differential IgG subclass responses to rBCG012 (Fig. 2b), with IgG1 predominating ($p< 0.01$) followed by IgG2a ($p< 0.01$), IgG3 ($p< 0.05$) and IgG2b ($p< 0.05$).

Reactivity of antibodies from rBCG-immunized with the SE22 protein on the merozoites

The IFA was carried out on *P. falciparum* infected erythrocytes using sera obtained from the BCG and rBCG012 immunized mice

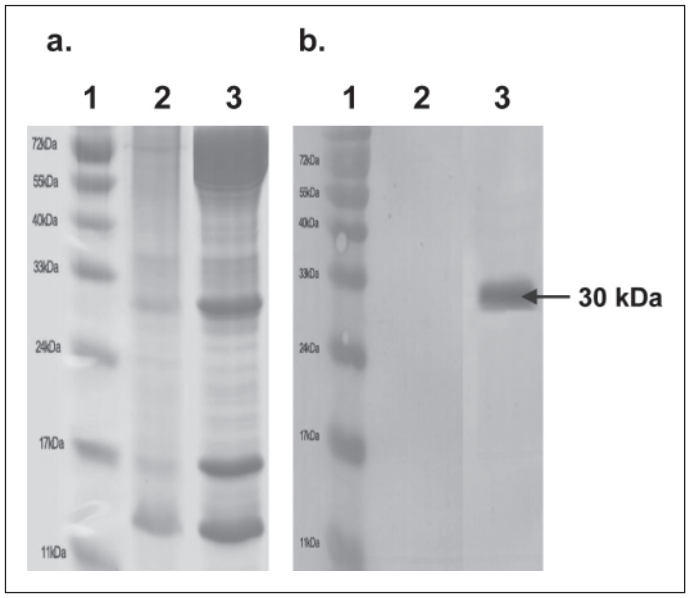

Figure 1. SDS-PAGE (a) and Western blot (b) analyses of rBCG012 using SE47 mAb. Lane 1, pre-stained protein marker; lane 2, parent BCG (negative control); lane 3, rBCG012 expressing SE22 (size ~30 kDa)

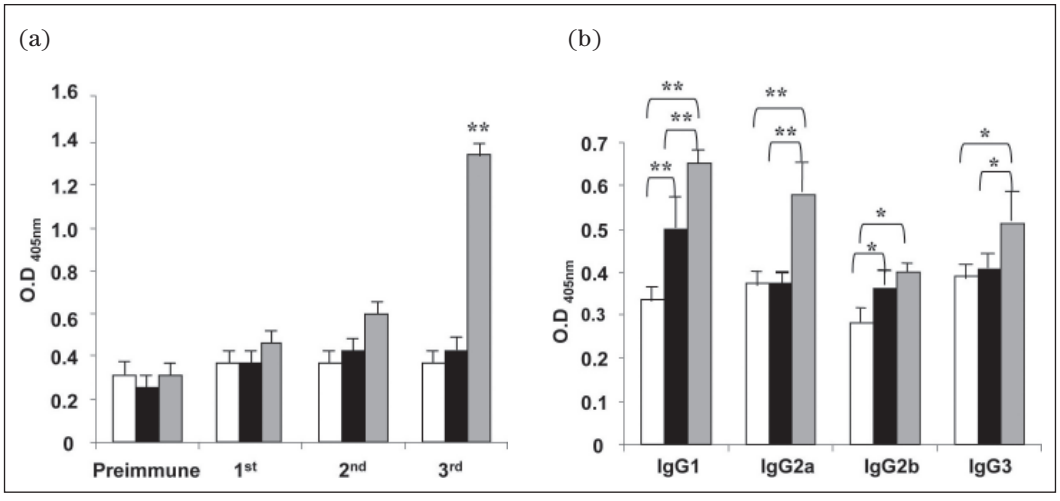

Figure 2. (a) The mean optical densities (OD_{415nm}) of total serum IgG from immunized mice against SE22 at different stages of vaccination. The bar chart represents the specific IgG levels against SE22 in the sera of mice immunized with: saline (white bars), BCG (black bars), or rBCG012 (grey bars), respectively. The results are expressed as mean OD±SD in each group of five mice performed in triplicates. Statistical analysis was performed using the Mann–Whitney test.**$p<0.01$ denote significant difference compared to saline or BCG

(b) Profile of specific IgG subclass responses to SE22 in mice vaccinated with saline (white bars), BCG (black bars), or rBCG012 (grey bars) from sera of 3-week post-third immunization. The results are expressed as mean OD±SD in each group of five mice performed in triplicates. Statistical analysis was performed using Mann–Whitney test. *$p<0.05$ and **$p<0.01$ denote significant difference compared to saline or BCG

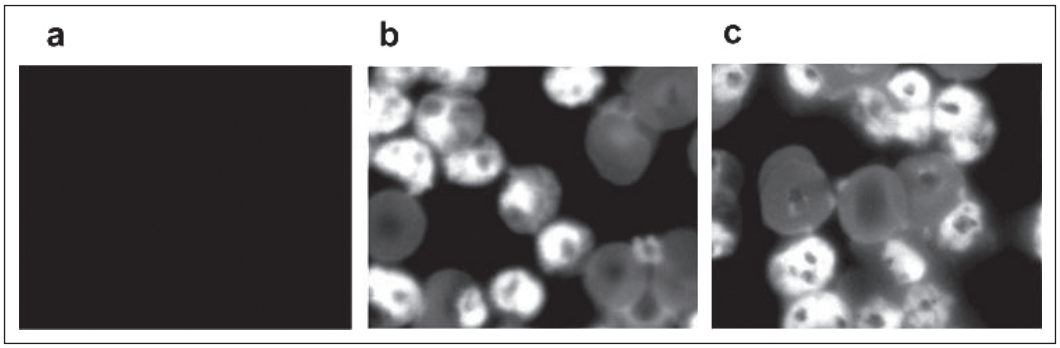

Figure 3. IFA using (a) preimmune serum, (b) SE47 mAb and (c) serum from rBCG012-immunized mice showing labeling of fixed 3D7 *P. falciparum* merozoites

as well as sera from unimmunized mice to determine the reactivity of the rBCG-immunized sera with the native protein on the *P. falciparum* merozoites. Microscopic examination showed that FITC fluorescence was detected on the merozoites when reacted with the serum samples from rBCG012 vaccinated mice and the control antibody SE47 specific IgG (Fig. 3). However, no reactivity was observed on the serum samples from unimmunized and BCG vaccinated mice. As shown in the Fig. 3, the sera of rBCG012-immunized mice were reactive with schizonts and trophozoites of the infected erythrocytes.

Intracellular cytokines of mice immunized with rBCG

A three colour flow cytometric analysis was performed to determine the proportions of $CD4^+$ and $CD8^+$ T-cells that express IL-2, IL-4 and IFN-γ cytokines in response to SE22 stimulation (Fig. 4). *In-vitro* stimulation of splenocytes from the rBCG012-immunized mice showed IFN-γ, IL-2 and IL-4 secretions in $CD4^+$ T-cells were detectable. The rBCG012-immunized mice secreted IFN-γ>IL-2>IL-4 higher than the control groups. Similarly, the expression levels of IL2, IL4 and IFNγ by the $CD8^+$ cells in rBCG012-immunized mice were also higher than the control groups. The level of cytokines secreted by the $CD8^+$ T-cells in the rBCG012 immunized group was IL-2>IFN-γ>IL-2.

DISCUSSION

The SERA of *P. falciparum* is one of the most promising blood stage malarial vaccine candidates. The 47 kDa N-terminal domain of SERA has been implicated in facilitating merozoite invasion of erythrocytes. Antibodies raised against this candidate molecule confer significant levels of protection in *in-vivo* and *in-vitro* systems (Banyal & Inselburg, 1985; Inselburg *et al.*, 1991, 1993 a and b; Pang & Horii, 1998; Pang *et al.*, 1999). Previous studies in Uganda, one of the malarial highly endemic areas, have reported significant increase in anti-SERA specific antibody in adults and children (Okech *et al.*, 2001, 2006). The most recent epidemiological study by Horii *et al.* (2010) showed that a modified version of SERA protein called SE36 showed that this vaccine was safe, well-tolerated and highly immunogenic when tested using non-human primates, and also a human Phase Ia clinical trial.

The entire 47 kDa N-terminal domain of SERA may not be necessary to confer protection. In the study carried out by Puentes *et al.* (2000) seven high erythrocyte binding non-overlapping 20-residue-long peptides within the whole SERA protein of FRC3 strain were identified. The conserved peptides 6725, 6733 and the polymorphic 6727 high binding peptides from SERA bind to the proteins located in the erythrocyte membrane. As

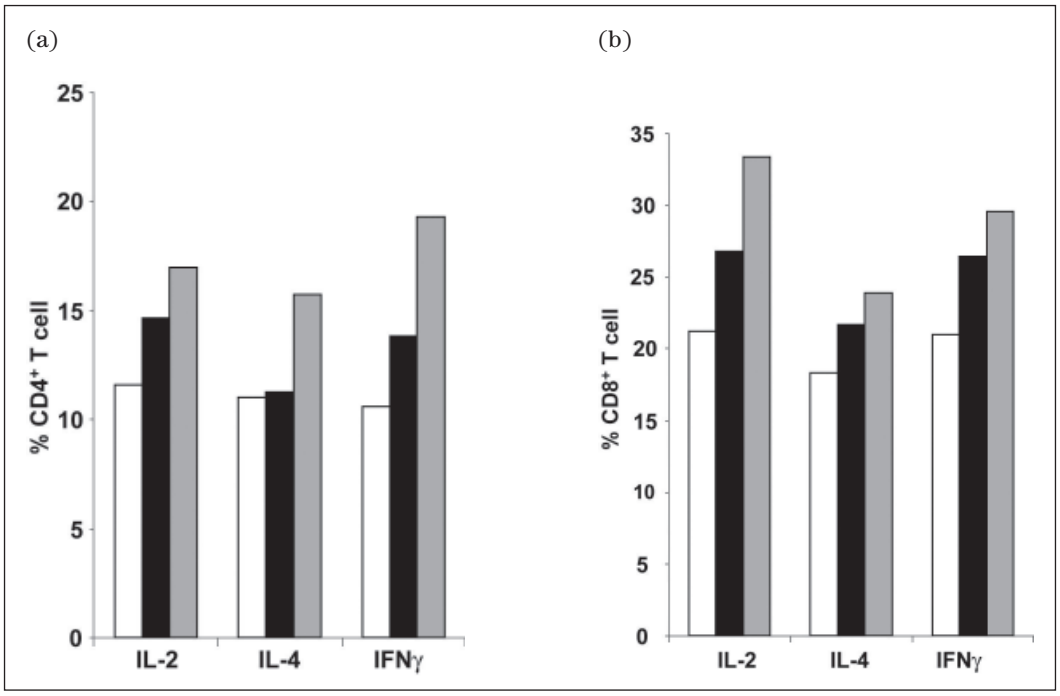

Figure 4. Pooled (a) $CD4^+$ and (b) $CD8^+$ splenocytes obtained from mice immunized with saline (white bars), BCG (black bars) and rBCG012 (grey bars) secreting IL-2, IL-4 and IFN-γ upon *in-vitro* stimulation with 5 µg/ml SE22 antigen

reported by Weber *et al.* (1987), sera from **FESNSGSLEKK**KYVKLPSNG peptide-immunized mice recognized SERA and this peptide shares part of the same sequence (in bold face type) with the high-binding peptide 6725 (Puentes *et al.*, 2000). It was suggested that there is a possibility that the 6725 high binding peptide is very hydrophilic and is exposed on SERA surface. The importance of identifying such high specific binding erythrocyte SERA proteins implies that these proteins may be important in interacting with the erythrocyte membrane during schizont rupture, merozoite liberation and reinvasion of the erythrocytes. Therefore in this study, a synthetic 22 kDA SERA which included the specific binding activity peptides spanning from peptide 6723 to 6728 was synthesized.

At the asexual blood stages, T-cell immunity is limited due to the lack of MHC I and MHC II expression in erythrocytes. The immune response therefore is predominantly dependent on antibody-associated processes where antibodies will help to neutralize the merozoites and prevent the invasion of erythrocyes (Holder *et al.*, 1999). In this study, significant levels of SE22-specific IgG were detected following immunization and boosting with rBCG012. The use of live BCG provides a stronger effect on immune regulation and as a live attenuated vaccine, BCG has the capability of multiplication hence reducing the need for boosting. To some degree, the higher the dosage of BCG vaccinated, the more extensive the Th1 response induced. Griffin *et al.* (1999) observed that booster immunizations with live BCG gave significant protection against experimental infection and disease caused by virulent *M. bovis* compared to single immunization with live BCG. Many studies have shown increased levels of antibody and T cell responses after booster doses (Bharadwaj *et al.*, 1998; Sun *et al.*, 2003; Wang *et al.*, 2004, 2005; Nurul & Norazmi, 2011). Therefore, in this study, boosting is needed to recall the memory cells and allow the immune system to mount a stronger secondary response on a second encounter of SE22.

The predominant IgG subclasses generated against SE22 were IgG1 and IgG2a although significant levels of IgG3 and IgG2b were also detected. The rBCG012 could therefore stimulate both Th1 and Th2 responses. Gor *et al.* (1998) showed that Balb/c mice infected with the murine malarial parasite, *Plasmodium vinckei* demonstrated a potent Th1 response where high IgG2a against $SERA_{Vin}$-1, 2 and 3 protease domains were produced. Sakai *et al.* (1999) found that DNA immunization against SERA induced the production of IgG2a and stimulated the Th1 response. Moreover, previous studies also showed that anti-SERA IgG2a, IgG2b and IgG3 are more potent than anti-SERA IgG1 in *in-vitro* parasite growth inhibition assays (Pang & Horii, 1998; Pang *et al.*, 1999). Matsumoto *et al.* (2001) produced an rBCG which expressed MSP-1 from *Plasmodium yoelii* showing enhancement of IgG2a production. However, Belperron *et al.* (1999) showed that a DNA vaccine containing SERA of *P. falciparum* and administered by gene gun immunization stimulated Th2 response and generated high IgG1. Smith & Taylor-Robinson (2003) reported that parasite-specific IgG1 and IgG2a are associated with protective immunity to blood stage malarial infection in mice. Studies carried out on blood stage antigens mostly showed high production of IgG2a and biasing immunity to Th1 responses which may ultimately play a primary role in protection (Gor *et al.*, 1998; Pang & Horii, 1998; Pang *et al.*, 1999; Matsumoto *et al.*, 2001; Zheng *et al.*, 2002b). This is associated with the inflammation during the infection by blood stage malarial parasites (Grau & Behr, 1994). More recently, Iriemenam *et al.* (2009) suggested that antibodies of certain IgG subclasses might aid in the control of clinical manifestations of malaria in individuals resident in endemic areas i.e. higher levels of IgG1 and IgG3 antibodies were more associated with protection than lower levels.

The proliferative response of rBCG012-vaccinated splenocytes was increased by about 7- to 8-fold compared to those vaccinated with either saline or BCG when stimulated with SE22 (results not shown). Analyses of intracellular cytokine expression revealed that splenocytes from rBCG012-vaccinated mice resulted in higher percentages of $CD4^+$ and $CD8^+$ cells expressing IL-2, IL-4 and IL-10 when stimulated with SE22. Troye-Blomberg *et al.* (1994) reported that in rodent malarias, the Th1 cells are important for controlling infection in the early phases while the Th2 cells which produce IL-4 and IL-10 are important for parasite clearance in the later stages of infection. BCG is also known to induce predominantly Th1 type immunity (Kaufmann & Andersen, 1998). The rBCG expressing the CS protein and MSP1 of *P. yoelii* (Matsumoto *et al.*, 1998); CSP and MSA 2 of *P. falciparum* (Zheng *et al.*, 2002a and b) showed predominantly Th1 response. The production of IFN-γ and IL-2 by $CD4^+$ T cells may also enhance the proliferation of $CD8^+$ cytotoxic lymphocytes (O'Donnell *et al.*, 1994; Young *et al.*, 2002). The IFN-γ could mediate cellular immunity by activating macrophages and enhance killing of parasites (Matsumoto *et al.*, 1998; Toore *et al.*, 2002).

Since mature RBCs do not express MHC class I or II antigens, it is difficult to predict a direct role for T cells in protective immunity against the blood stage of the malaria parasite (Aidoo & Udhayakumar, 2000). Although the blood-stage parasite is not a target for T cell attack, previous studies showed that T cells and cytokines play a crucial role in the protective immunity to blood-stage malaria (de Souza *et al.*, 1996; Plebanski & Hill, 2000). An increasing body of evidence from both murine models and human studies indicates that T cells, monocytes, and cytokines may be important in determining the level of malaria parasitemia and rates of clearance (Taylor-Robinson, 1995; Winkler *et al.*, 1998; Jason *et al.*, 2001). It would be interesting to determine the presence of T cell epitopes within the SE22 antigen and whether they influence the level and types of antibodies being produced.

The IFA results showed that parasite infected erythrocytes could be detected by the sera from the rBCG012 vaccinated mice. This indicates that the rBCG could induce SE22 specific antibodies in mice and react against native SE22 protein expressed on

the parasites. The expression also showed that the SE22 was localized in the parasitophorous vacuole at the late trophozoite and schizont stages. This result confirms previous reports that SERA is localized in the parasitophorous vacuole (Perkin & Ziefer, 1994; Pang & Horii, 1998). SERA has been reported to localize on the merozoite surface and has been proposed to play an important role in merozoite release. It binds to the lipids of the parasitophorous vacuole membrane or erythrocyte plasma membrane and it facilitates membrane lysis to facilitate merozoites to invade the erythrocytes (Perkin & Ziefer, 1994). Pang *et al.* (1999) reported that the SE47'-specific IgG could inhibit parasite growth *in-vitro*. The SE47'-specific IgG binds to the rupturing schizont and causes the agglutination of the parasite cells, thus inhibiting the free merozoites from being released and blocking merozoite invasion. Since rBCG012 induced SE22 specific antibodies, further studies are necessary to directly determine the protective effect of rBCG012 vaccine.

In conclusion, the rBCG candidate vaccine expressing SE22 of *P. falciparum* induced significant humoral and cellular responses in mice. Moreover, the sera obtained from rBCG-immuniced mice also recognized and reacted with fixed *P. falciparum*. Thus, the use of rBCG approach may pave the way for the development of a live BCG-based malaria vaccine.

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