BRIEF REPORT

ELEVATED T-CELL RESPONSE IN LIVER OF MICE VACCINATED WITH ATTENUATED KOREA VACCINIA VIRAL VACCINE EXPRESSING *PLASMODIUM VIVAX* CIRCUMSPOROZOITE PROTEIN

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Abstract. Relapse and recurrence of vivax malaria is still a major issue in temperate regions including Korea. Recombinant attenuated vaccinia virus KVAC103 expressing circumsporozoite protein (CSP), one of the main antigenic proteins of *Plasmodium vivax*, (KVAC-PvCSP) was generated and evaluated for its potential as an anti-malarial vaccine. Mice were subcutaneously inoculated twice with KVAC-PvCSP, with a three-week interval between injections, and cellular as well as humoral immune responses, including memory B cell response, were examined. Serial inoculation of KVAC-PvCSP elicited strong IgG production in mice. Moreover, $CD3^+$, $CD4^+$ and $CD8^+$ T-cells were increased by vaccination in mouse hepatocytes, but not in splenocytes. Thus, serial KVAC-PvCSP vaccination elicited $CD4^+$ and $CD8^+$ T-cell responses in the liver of mice. These results suggest KVAC103-based vaccination may be useful for targeting pre-erythrocytic stages of vivax malaria.

Keywords: *Plasmodium vivax*, circumsporozoite protein, malaria, vaccine, vaccinia virus

INTRODUCTION

Malaria caused by *Plasmodium vivax* infection is the most geographically widespread human malaria, mostly prevalent in Latin America and Southeast Asia (Howes *et al*, 2016). During the past decades, vivax malaria caused significant human and economic losses, in Korea, as well as the rest of the world (Bahk *et al*, 2018). Although lethality is lower than that of other forms of the disease, vivax malaria is still a major clinical concern due to infection relapse and recurrence,

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a hallmark of this disease, especially in temperate regions (Chu and White, 2016). Long incubation period and recurrence of vivax malaria are caused by presence of hypnozoites in infected liver (Dayananda *et al*, 2018).

Vaccines capable of inducing $CD8^+$ T-cell responses in the liver have a potential to halt this disease at the pre-erythrocytic stage and are considered a promising approach for the prevention of vivax malaria (Nardin *et al*, 1999; Tsuji and Zavala, 2001; Arevalo-Herrera *et al*, 2002; Suphavilai *et al*, 2004; Fonseca *et al*, 2018). In this regard, circumsporozoite protein (CSP), the major surface protein of *Plasmodium* sporozoite, is widely regarded as a potential vaccine candidate target (Pinzon-Ortiz *et al*, 2001; Singh *et al*, 2007; Coppi *et al*, 2011). Notably, evidences supporting a role of CSP-specific antibodies and T-cells in malaria protection have been reported (Sun *et al*, 2003; Mishra *et al*, 2012; Vijayan *et al*, 2012; Foquet *et al*, 2014; Huang *et al*, 2014). RTS, S/AS01 subunit vaccine comprising *P. falciparum* CSP, hepatitis surface antigen and AS01 adjuvant has shown protective efficacy in a Phase 3 study of African children (Cohen *et al*, 2010; Tinto *et al*, 2019). Studies on anti-vivax malarial subunit vaccines consisting parts of CSP should be carried out.

A highly attenuated vaccinia virus strain, KVAC103, initially developed for a third-generation smallpox vaccine (Lee *et al*, 2016), was modified to express *P. vivax* CSP (PvCSP) and evaluated as a possible effective strategy to induce $CD8^+$ T-cell response against PvCSP. KVAC103-based vaccine expressing PvCSP demonstrated ease of production and potential for pre-erythrocytic stages of vivax malaria.

MATERIALS AND METHODS

Production of viral vaccines

Fragments of PvCSP-K (GenBank accession no. AJ278611) and PvCSP-Sal1 (GenBank accession no. DQ156134) cDNA,

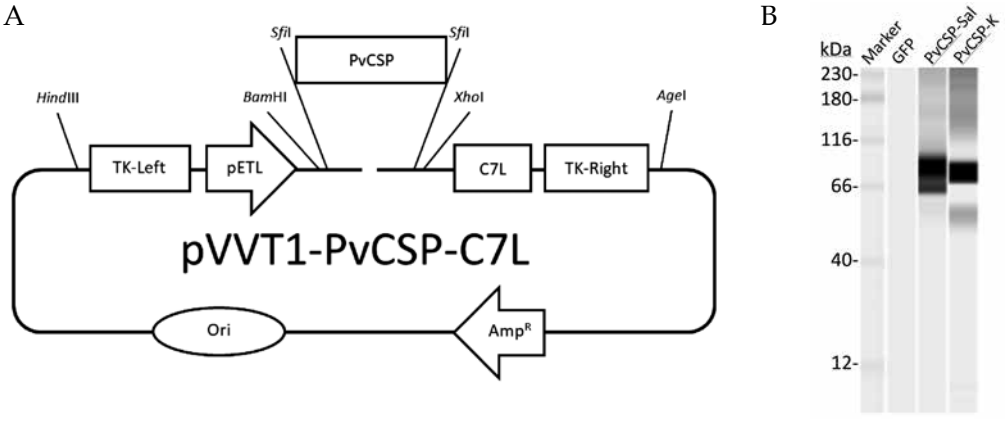

Fig 1- Shuttle vector for production of PvCSP vaccine (A) and immunoblot of KVAC-PvCSP vaccinia viruses produced from Vero cells; (B). Vero cells were co-transfected with individual cloned shuttle vector and vaccinia virus KVAC103 and transfected Vero cells expressing recombinant vaccinia viruses, KVAC- PvCSP-K and KVAC-PvCSP-Sal1, were identified by immunoblotting. Marker: protein molecular weight markers; GFP: recombinant green fluorescence protein; PvCSP-Sal: recombinant PvCSP-Sal protein; PvCSP-K: recombinant PvCSP-Sal protein.

both devoid of GPI-anchor region, were amplified and introduced into *Sfi*I site of pVVT1-GFP-C7L shuttle vector developed by Lee *et al* (2016) (Fig 1A). Vero cells (ATCC, Manassas, VA, USA) were co-transfected with individual cloned shuttle vector and vaccinia virus KVAC103 (Lee *et al*, 2016). Following homologous recombination, transfected Vero cells expressing PvCSP-K and PvCSP-Sal1were identified using immunoblotting (Fig 1B) and recombinant vaccinia viruses, KVAC-PvCSP-K and KVAC-PvCSP-Sal1, were isolated (Lee *et al*, 2016) and stored at -80°C until used.

Vaccination and immunological assays

For immunological assays, 1×10^7 PFU of KVAC-PvCSP-K, KVAC-PvCSP-Sal1 and negative control vaccine KVAC-GFP were separately injected subcutaneously into BALB/c mice twice, with a three-week interval. Two weeks following the last booster inoculation, prevalence of total immunoglobulins, as well as isotypes, was measured by conventional ELISA (Engvall, 1972). Further characterization of vaccine-induced antibody responses against PvCSP-Sal1 was carried out by ELISA-based assessment of immunoglobulin subsets. PvCSP-specific T-cell responses induced by vaccination were evaluated by examining $CD3^+$, $CD8^+$, and $CD4^+$ T cells from liver and spleen of vaccinated BALB/c mice using intracellular cytokine staining and flow cytometry (Picot *et al*, 2012). Population of IFN-γ-producing T cells were assessed using an *ex vivo* ELISPOT assay (Darwin Bio, Seoul, Korea) was performed following re-stimulation of splenocytes with each of the 11 selected H-$2K^b$-restricted immunodominant individual peptides (10-mer) (Petron, Daejeon, Korea) predicted to contain T-cell epitopes of PvCSP-Sal1 (IEDB Analysis Resource, http://www.iedb.org). Examination of memory B-cell activation in vaccinated BALB/c mice was conducted by immunizing animals as described above, and after three months PBMCs were isolated, cultured, stimulated with lipopolysaccharide, phorbol 12-myristate 13-acetate and ionomycine (Sigma Aldrich, St Louis, MO), and then memory B cell responses were analyzed by IgG ELISA against recombinant PvCSP-Sal1 protein (Cosmogenetech, Seoul, Korea).

Statistical analysis

Significance of differences between groups was evaluated using Student *t*-test and relationships between variables were examined by simple linear regression. Statistically significant difference is accepted at p-value <0.05.

RESULTS

Humoral immune response

Total IgG production against recombinant PvCSP-K and PvCSP-Sal1 protein was elevated in vaccinated mice, with no significant difference in mean titer between KVAC-PvCSP-Sal1 and KVAC-PvCSP-K vaccinated group (Fig 2A). All tested subtypes, except IgG2a, are significantly increased compared to control mice, IgG2b being the most prevalent subtype, indicating occurrence of Th1-biased response in vaccinated mice (Fig 2B).

Cellular immune response

No significant increase in effector T-cell number was observed in splenocytes (Fig 3), but in hepatocytes, number of PvCSP-Sal1-specific $CD3^+$ and $CD8^+$ T-cell was increased by 25.6% and 33.4% respectively compared to control group. Among the tested peptides, *ex vivo* ELISPOT assay revealed five peptides eliciting IFN-γ-producing T-cells, ranging

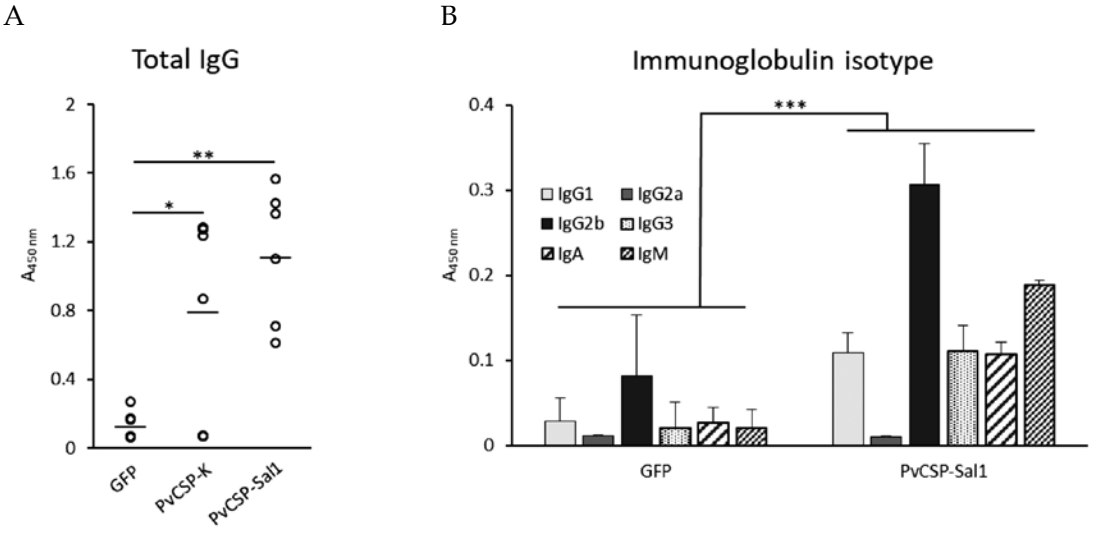

Fig 2- Humoral immune response induced in mice vaccination with KVAC-PvCSPs. KVAC-PvCSP-K, KVAC-PvCSP-Sal1 and negative control vaccine KVAC-GFP (1×10^7 PFU) were separately injected subcutaneously into BALB/c mice (n =6, 6 and 8, respectively) twice, with a three-week interval. Two weeks following the last booster inoculation, levels of total IgG (short horizontal line indicates mean value) (A) and immunoglobulin isotypes (mean ± SD) (B) to each vaccination were measured by ELISA. GFP: recombinant green fluorescence protein; PvCSP-Sal: recombinant PvCSP-Sal protein; PvCSP-K: recombinant PvCSP-Sal protein. *p-value < 0.05.

from 84 to 190 IFN-γ SFU / 10^6 cells (Table 1). Overall, moderate T-cell responses were observed in vaccinated mice with no significant differences among all three vaccinated mice groups (Fig 4).

DISCUSSION

Modified vaccinia virus Ankara (MVA) vectors have been developed for generating protective immunity against various antigens in animal models and in humans (Gilbert, 2013; Ewer *et al*, 2016; Sebastian and Gilbert, 2016). Although MVA vectors have been used for the development of anti-malarial vaccines, more efficient viral vaccine platforms are needed (Cottingham and Carroll, 2013). KVAC103 vaccinia virus used in this study was initially developed to overcome the difficulties in developing vaccines against some infectious diseases and cancers (Lee *et al*, 2016). Difficulties in developing vaccines against malaria can be reduced by using KVAC103-based platform, which has the potential to induce effective humoral and cellular immune responses.

High efficiency in expansion and activation of $CD4^+$ and $CD8^+$ T cells is crucial for *P. vivax* removal in liver, and strong antibody prevalence is required for clearance of sporozoites by opsonization and phagocytosis (Bertolino and Bowen, 2015). Prime-boost vaccination using different recombinant viral vectors, such as human or chimpanzee adenovirus MVA (expressing thrombospondin-related anonymous protein (TRAP), apical membrane antigen-1 (AMA-1) and other antigenic candidates) and adenovirus, have been tested as anti-malarial vaccines

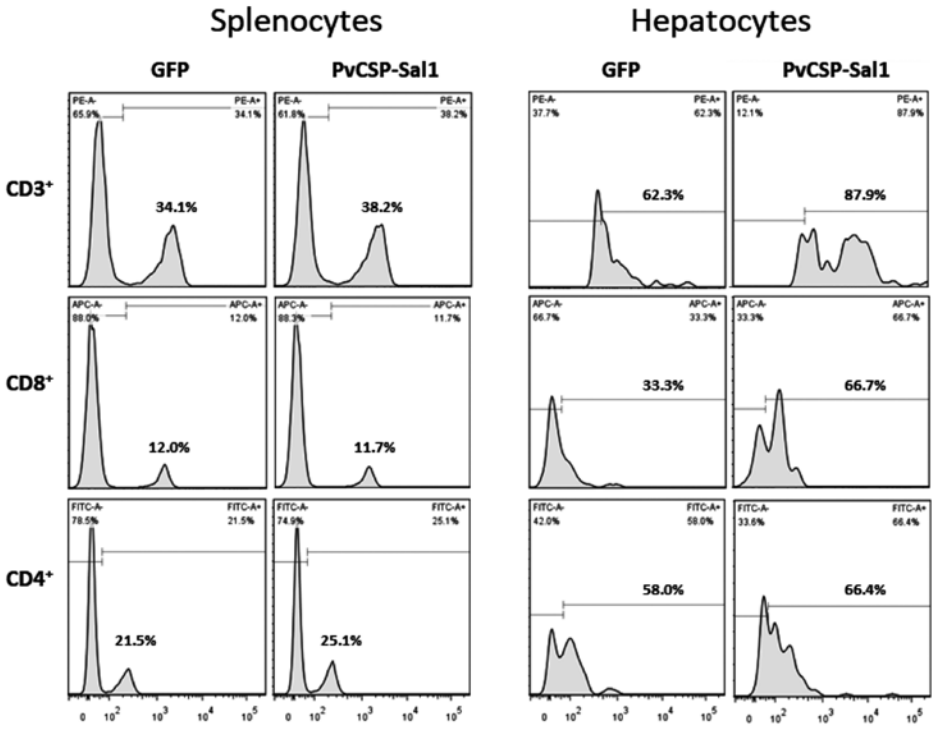

Fig 3 - Cellular immune response induced in mice vaccinated with KVAC-PvCSPSal1. Inoculation protocols are described in legend to Fig 2. Numbers of CD3+, CD8+ and CD4+ T cells in spleen and liver of vaccinated BALB/c mice were measured by flow cytometry. GFP: recombinant green fluorescence protein; PvCSP-Sal1: recombinant PvCSP-Sal protein.

Table 1
H-2K^b T cell epitopes mapped to *Pv*CSP-Sal1.

Peptide number	Sequence	IFN-γ spot forming U*
5	CTMDKCAGIF	84
26	KNFILLAVSS	160
35	NFNNVDASSL	118
49	VGTEWTPCSV	190
50	VNFNNVDASS	189

*Based on *ex vivo* ELISPOT assay.

through Phase I and Phase IIa trials with promising results (Hill *et al*, 2010; Rollier *et al*, 2011; O'Hara *et al*, 2012). However, only a limited number of prime-boost vaccination for vivax malaria have been tested (Bauza *et al*, 2014; de Cassan *et al*, 2015). New modified human adenoviral vectors expressing various subunit vaccine candidates are under development (data not published), which can be used in combination with KVAC103 for development of new prime-boost vaccines.

The present study demonstrates immunization of mice with recombinant vaccinia KVAC103 vector carrying cDNA of two different fragments of PvCSP resulted in cellular immunity in hepatocyte as well as strong humoral immune responses. These results imply the potential of KVAC103 carrying PvCSP

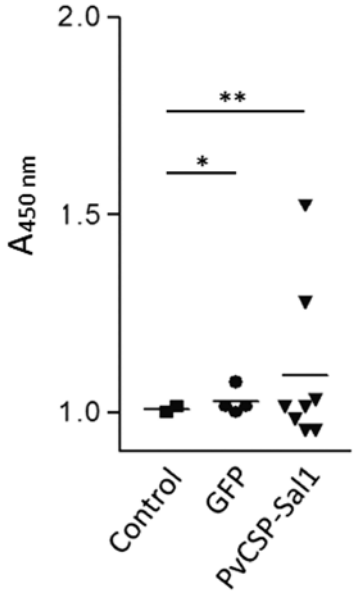

Fig 4- Memory B-cell responses in mice vaccinated with KVAC-PvCSPSal1. Inoculation protocols are described in legend to Fig 2. Levels of memory B cell were determined by ELISA three months after last boosting vaccination. Short horizontal line indicates mean value. Control: recombinant PvCSP-Sal protein reacted with sera from unvaccinated mice; GFP: recombinant green fluorescence protein; PvCSP-Sal1: recombinant PvCSP-Sal protein.

fragment as an effective vaccine for vivax malaria. The five peptides eliciting IFN-γ-producing T-cells need to be investigated further to construct a more defined subunit vaccine. These findings highlight the potential of KVAC103-based vaccines as a strategy to protect against pre-erythrocytic stage vivax malaria.

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