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Korea vaccinia viral vectored vaccine expressing 33 kDa fragment of *Plasmodium vivax* merozoite surface protein 1 elicited strong humoral immune responses in mice

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마우스에서 강력한 항체 반응을 유도하는 삼일열 원충 merozoite surface protein-1 33 kDa 분획 발현 백시니아 바이러스 백신주

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Vivax malaria is the most widely distributed human malaria in the world. Relapse and recurrence in temperate regions are major issues related to vivax malaria. Until now, no adequate vaccines for vivax malaria were developed. In this study, a recombinant attenuated Korea vaccinia virus (KVAC103) expressing the 33 kDa fragment of merozoite surface protein-1 ($PvMSP1_{33}$), the most abundant surface protein of *Plasmodium vivax*, was generated and evaluated for its potential as an anti-malarial vaccine. The $PvMSP1_{33}$-expressing virus (KVAC-PvMSP133) was generated in Vero cells by homologous recombination with KVAC103 and then purified. The vaccine was inoculated twice with 3-week intervals into the mice subcutaneously. Next, cellular and humoral immune responses in the mice were examined. KVAC-$PvMSP1_{33}$ elicited weak $CD3^+$, $CD4^+$, and $CD8^+$ T-cell responses in the spleen of vaccinated mice. However, the production of $PvMSP1_{33}$-specific IgG was significantly increased in the peripheral blood of the mice. Among the immunoglobulin subtypes, IgG2b showed the strongest effects. Serial KVAC- $PvMSP1_{33}$ vaccination elicited strong humoral immune responses rather than cellular immune responses in mice. KVAC103 vector expressing Plasmodium antigen(s) and antibodies elicited by the vaccination can be used as a platform for producing protective anti-malarial vaccine.

Keywords: merozoite surface protein-1, vaccine, vaccinia virus, vivax malaria

Malaria is caused by infection with parasites from the genus *Plasmodium*. Five species of parasites, *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*, can infect humans through anopheline mosquito vectors. Among the human malaria parasite species, *P. vivax* is the most geographically widespread and is mostly prevalent in Latin America and Southeast Asia (Howes *et al*., 2016). In Korea, vivax malaria, which re-emerged in the early 1990s, has been endemic in the northern part of Gyeonggi-Do province for more than two decades. Although the lethality of vivax malaria is much lower than that of other forms of human malaria, it remains a major clinical concern because infection relapse and recurrence are common in temperate regions, including in Korea (Chu and White, 2016). Hypnozoites, which are dormant forms of the parasite in the infected liver, are thought to cause long incubation periods of vivax malaria in temperate regions.

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Merozoite surface protein 1 (MSP1) is the most abundant surface protein of merozoites of *Plasmodium* parasites (Holder *et al*., 1999). At the point of merozoite invasion of a red blood cell, the C-terminal 42 kDa protein ($MSP1_{42}$) is cleaved to produce a 33 kDa ($MSP1_{33}$) and 19 kDa ($MSP1_{19}$) fragment (Balcakman *et al*., 1991; Blackman and Holder, 1992). $MSP1_{33}$ is shed from the merozoite surface and released into the blood plasma, while $MSP1_{19}$ which contains the glycosylphosphatidylinositol moiety remains on the surface of the invading merozoite (Blackman *et al*., 1990).

$MSP1_{42}$ and its components have been leading candidates of blood stage human malaria vaccines. $MSP1_{42}$ and $MSP1_{19}$ can induce protection by inducing protective antibodies (Burns *et al*., 1989; Chang *et al*., 1996; Hirunpetcharat *et al*., 1997; Tian *et al*., 1998; Egan *et al*., 1999; Vukovic *et al*., 2002; Wipasa *et al*., 2002b; Okech *et al*., 2004; Perraut *et al*., 2005). However, the role of $MSP1_{33}$ in this protection is unclear. Some previous studies showed that protective antibodies are specific for $MSP1_{19}$ and $MSP1_{33}$ and can provide additional T helper epitopes (Tian *et al*., 1998; Wipasa *et al*., 2002a, 2002b).

No adequate T helper epitopes have been identified for stimulating antibody production in $MSP1_{19}$ (Udhayakumar *et al*., 1995; Tian *et al*., 1996). Therefore, studies of $MSP1_{33}$ have primarily focused on finding T cell epitopes (Lee *et al*., 2001; Malhotra *et al*., 2008). Studies indicated that T cell responses to epitopes of $MSP1_{33}$ delayed the growth of *P. yoelii in vivo* in the absence of antibodies (Wipasa *et al*., 2002a). $MSP1_{33}$ may also have vaccine potential as observed for $MSP1_{42}$ and $MSP1_{19}$. However, there are little evidences whether $MSP1_{33}$ can induce a protective antibody response against human malaria.

Modified vaccinia virus Ankara (MVA) vectors have been developed to generate protective immunity against various antigens in animal models and humans (Cottingham and Carroll, 2013; Gilbert, 2013; Sebastian and Gilbert, 2016). Although modified vaccinia virus Ankara vectors have been broadly used for the development of anti-malarial vaccines, more efficient viral vaccine platforms are needed.

In the present study, a highly attenuated vaccinia virus strain, KVAC103, initially developed as a third-generation smallpox vaccine (Lee *et al*., 2016), was modified to express *P. vivax* $MSP1_{33}$ ($PvMSP1_{33}$). Our aim was to evaluate KVAC103-based vaccination as an effective strategy for inducing T cell and antibody responses against $PvMSP1_{33}$.

Materials and Methods

Generation of viral vectored vaccine

To generate a KVAC103-vectored viral vaccine expressing $PvMSP1_{33}$, cDNA portions of $PvMSP1_{33}$ Belem (GenBank Acc. No. AF435594) were cloned into *Sfi*I sites of the shuttle vector pVVT1-GFP-C7L (Lee *et al*., 2016) (Fig. 1A). Next, the produced shuttle vector pVVT1-$PvMSP1_{33}$-C7L and the vaccinia virus KVAC103 (Lee *et al*., 2016) were co-introduced into Vero cells (ATCC). Following homologous recombination in the cells, recombinant vaccinia virus named as KVAC-$PvMSP1_{33}$ was produced. The secreted KVAC-$PvMSP1_{33}$ was harvested from the cell culture media confirmed by immunoblot analysis using anti-$PvMSP1_{33}$ antisera produced from BALB/c mice immnunized with the recombinant $PvMSP1_{33}$ (Fig. 1B).

Vaccination

For vaccination, 1×10^7 PFU of KVAC- $PvMSP1_{33}$ and negative control vaccine KVAC-GFP were injected subcutaneously into BALB/c mice (Koatech) twice at a 3-week interval. Two weeks after the final injection, 500 μl of peripheral blood was obtained from the retro-orbital plexus of each mouse. The blood was stored at 4°C overnight and then centrifuged at 10,000 × *g* for 10 min. The sera were harvested from the supernatant and stored at -70°C until use.

Immunological assay

To examine the humoral immune responses of the vaccinated mice, enzyme-linked immunosorbent assay (ELISA) was performed against the recombinant $PvMSP1_{33}$ protein. Briefly, a 96-well ELISA plate (SPL) was coated with recombinant $PvMSP1_{33}$ protein overnight, and then, the wells were washed twice with PBS-T. Sera from mice were diluted by 1:100 with PBS-T, and added to the wells. After incubation, the plate was washed as above. HRP-conjugated anti-mouse IgG (BD Biosciences) diluted by 1:1,000 was added to the wells and incubated. Color development was performed by reacting the plate with *o*-phenylenediamine (Merck KGaA). The optical densities of the plate were measured at a wavelength of 450 nm. Four weeks after the final injection, the mice were sacrificed. To prepare splenocytes, spleens were collected from the mice and homogenized thoroughly under aseptic conditions. After

incubation with red blood cell (RBC) lysis buffer (BD Biosciences), the splenocytes were washed repeatedly with PBS and RPMI 1640 media (Merck KGaA) by suspension and centrifugation, and then cultured in a 5% CO_2 incubator. To detect cytokine secretion, flow cytometry and intracellular cytokine staining analysis were performed. Briefly, splenocytes were stimulated with 1 mg/ml of recombinant $PvMSP1_{33}$ protein for 6 h in a 96-well plate. After washing, Fc receptors in the cells were blocked using anti-CD16/CD32 (BD Biosciences). Next, the cells were stained with surface-specific mouse $CD3^+$, $CD4^+$, or $CD8^+$ antibodies (BD Biosciences). The cells were then passed through a flow cytometer (BD Biosciences) and the data were analyzed.

Statistical analysis

The significance of differences between groups was evaluated using the Student *t*-test (SPSS ver. 18.0), and relationships between variables were examined by simple linear regression. Statistically significant difference is accepted at p-value < 0.05.

Results

Generation of viral vectored vaccine

$KVAC\text{-}PvMSP1_{33}$ was generated by homologous recombination between shuttle vector $pVVT1\text{-}PvMSP1_{33}\text{-}C7L$ and KVAC103 and secreted from Vero cells. Immunoblot using anti-$PvMSP1_{33}$ mouse antisera revealed 100 kDa band, which is originated from $PvMSP1_{33}$ and viral proteins, in the fraction of cell secretion (Fig. 1B). The band was not appeared in the fractions of cell lysate and cell secretion of KVAC-GFP used as a negative control.

T cell responses of the vaccinated mice

To examine $PvMSP1_{33}$-specific T cell responses in BALB/c mice inoculated twice with $KVAC\text{-}PvMSP1_{33}$, $CD3^+$, $CD4^+$, and $CD8^+$ T cells of splenocytes were assessed by T cell surface marker staining and flow cytometry. The proportion of $CD3^+$, $CD4^+$, and $CD8^+$ T cells in the splenocytes $KVAC\text{-}PvMSP1_{33}$-vaccinated mice was 40.9%, 25.5%, and 14.5%, respectively. Generally, the levels of $CD3^+$, $CD4^+$, and $CD8^+$ T cells were slightly higher, but not significant, in the splenocytes of mice

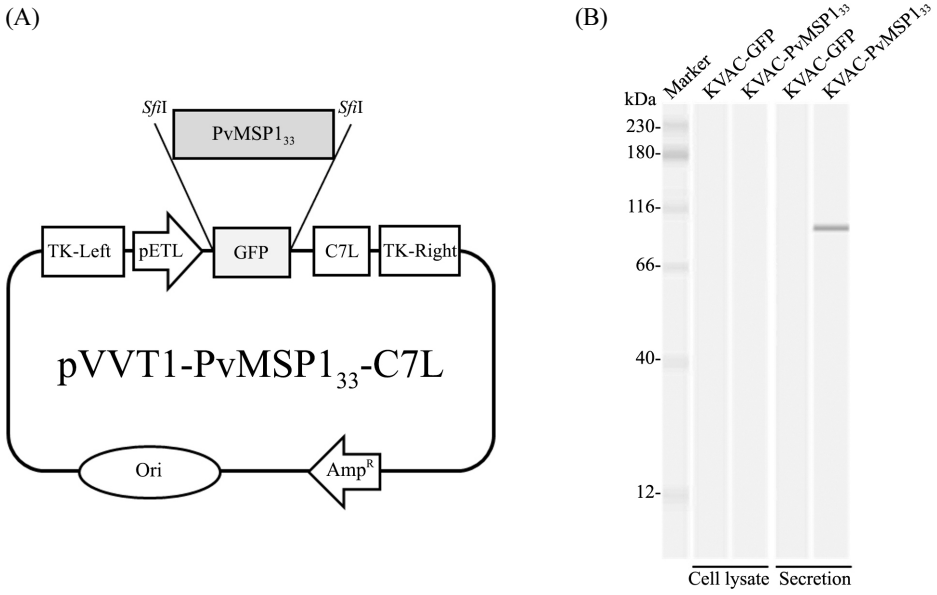

Fig. 1. Generation of KVAC-PvMSP1$_{33}$. (A) Construction of the shuttle vector for producing a vaccine expressing $PvMSP1_{33}$. GFP marker region in pVVT1-GFP-C7L was replaced by $PvMSP1_{33}$ sequence by *sfi*I double digestion. (B) Immunoblot of $KVAC\text{-}PvMSP1_{33}$ viral vaccine produced in Vero cells. Vero cells inoculated with shuttle vector and KVAC103 vector were cultured to produce viral vaccines. Then, cells and culture media were harvested and lysed to obtain vaccines using standard protocol elsewhere. Production of vaccines were confirmed by immunoblot using anti-$PvMSP1_{33}$ antisera produced from BALB/c mice immunized with the recombinant $PvMSP1_{33}$ protein. Virus produced using pVVT1-GFP-C7L shuttle vector, named as KVAC-GFP, was used as a negative control. Cell lysate, Vero cell lysate; Secretion, Vero cell culture media. Same results were obtained by duplicated experiments.

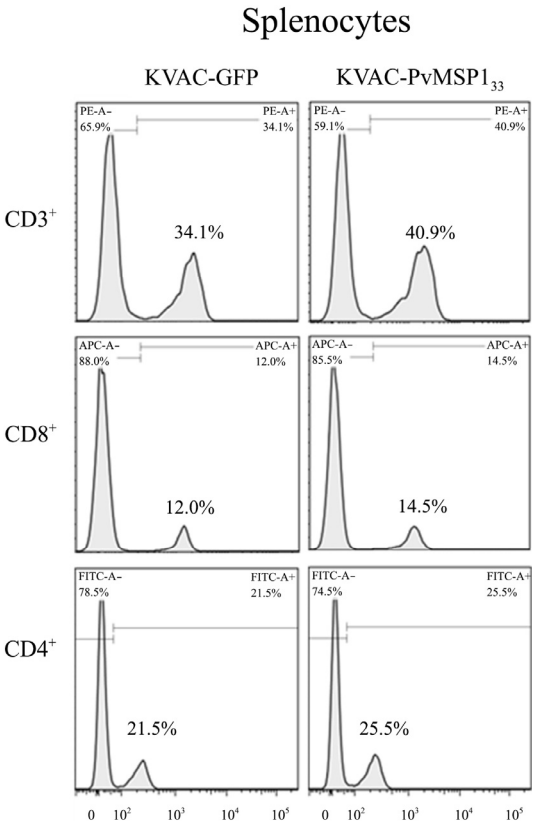

Fig. 2. Cellular immune responses induced by KVAC-PvMSP1$_{33}$. BALB/c mice (n = 5/group) were immunized with 10^7 pfu of KVAC-GFP or KVAC-PvMSP1$_{33}$. Splenocytic CD3$^+$, CD8$^+$, and CD4$^+$ T cell responses after stimulation with recombinant PvMSP1$_{33}$ protein were assessed by T cell surface staining. Proportions of elevated cell surface markers were calculated manually. Splenocytes from mice vaccinated with KVAC-GFP was used as a negative control. Similar results were examined by repeated experiments.

vaccinated with KVAC-PvMSP1$_{33}$ than those of mice vaccinated with KVAC-GFP (Fig. 2). Viral portion of KVAC-PvMSP1$_{33}$ seems to cause basal elevation of T cell responses.

IgG production of the vaccinated mice

ELISA revealed that total IgG production against recombinant PvMSP1$_{33}$ protein was significantly elevated in vaccinated BALB/c mice (Fig. 3A). To further characterize vaccine-induced antibody responses against PvMSP1$_{33}$, immunoglobulin subsets were assessed in the ELISA. All tested subtypes, except for IgG2a, were increased in vaccinated BALB/c mice (Fig. 3B). Among the tested immunoglobulin subtypes, IgG2b was the most prevalent, and IgM showed a remarkable increase.

Discussion

It has been known that sequence of MSP1$_{19}$ is well conserved and contains epitopes for IgG production, whereas MSP1$_{33}$ is highly polymorphic and contains relatively low antigenicity (Ahlborg *et al*., 2002; Yuen *et al*., 2007; Draper *et al*., 2008, 2009). Similar results were obtained from the study using Korean vivax malaria patient's sera (Dinzouna-Boutamba *et al*., 2016). Although MSP1$_{33}$ of murine malaria parasite *P. yoelli* was highly immunogenic and elicited levels of antibodies

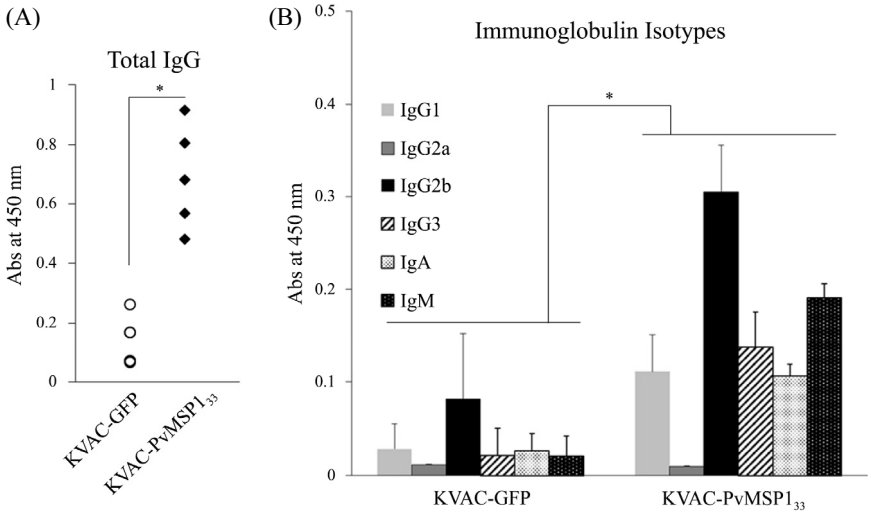

Fig. 3. Immunoglobulin responses of BALB/c mice induced by vaccination with KVAC-PvMSP1$_{33}$ examined by ELISA. Levels of total IgG (A) and immunoglobulin subtypes (B) to each vaccinations were revealed by absorbance at 450 nm of wavelength (* $P \leq 0.05$). Sera obtained from the mice and anti-mouse immunoglobulins were used as primary and secondary antibody for ELISA, respectively. Sera from mice vaccinated with KVAC-GFP was used as a control. Similar ELISA values were obtained by triplicated experiments.

to native MSP1, the antibodies were not protective (Ahlborg *et al*., 2002). Interestingly, several mice immunized with $MSP1_{33}$ revealed titers of all IgG subtypes that were comparable to or even higher than those in mice immunized with $MSP1_{19}$. The finding that *P. yoelii* $MSP1_{33}$ can elicits strong but non-protective antibody responses in mice prompted us to try to develop various vaccine flatform expressing *P. vivax* $MSP1_{33}$ with a strong antigenicity.

In this study, we examined the antigenicity and immunogenicity of $PvMSP1_{33}$ in mice by vaccinating them with a $PvMSP1_{33}$-expressing vaccinia viral vaccine. Briefly, KVAC-$PvMSP1_{33}$-vaccinated mice showed increased immunoglobulin subtypes and slightly elevated T cell responses. Most of all tested subtypes were significantly increased compared to in control mice, indicating the occurrence of Th1-biased responses in vaccinated mice. The vaccinica viral vector KVAC103 used in this study as a backbone of KVAC-PVMSP133 was used not only for the delivery of the antigen, but for the action as an adjuvant itself. As mentioned above, therefore, basal level of T cell activation could be caused by the KVAC-GFP. As IgM in the vaccinated mice was remarkably increased, general humoral immune responses against $PvMSP1_{33}$ may have been enhanced during vaccination. However, as there was not a significant increase of $PvMSP1_{33}$-specific splenic $CD4^+$ and $CD8^+$ T cells in the mice vaccinated with KVAC-$PvMSP1_{33}$, ways to improve the cellular immune responses following vaccination with $PvMSP1_{33}$ is quite confusing. To improve these weak cellular immune responses and enhance humoral immunity to prevent RBC invasion by merozoites, regimens for prime-boost vaccinations using a human adenoviral vector (Draper *et al*., 2008, 2009; Hill *et al*., 2010; Rollier *et al*., 2011; Ewer *et al*., 2016) and KVAC103 expressing $PvMSP1_{33}$ and/or other *P. vivax* antigens are being developed in our laboratory.

Modified vaccinia virus Ankara (MVA) vectors have been developed for generating protective immunity against various antigens in animal models and in humans (Gilbert, 2013; Sebastian and Gilbert, 2016). Although MVA vectors have been used for 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). Along with the results obtained in this study, 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.

Collectively, $PvMSP1_{33}$ vaccination elicited humoral immune responses rather than cellular immune responses. We demonstrated the potential of KVAC103-based vaccines expressing $PvMSP1_{33}$ as a strategy for preventing erythrocytic stages of vivax malaria. Inducing anti-$PvMSP1_{33}$ antibody can provide an advantage over using an erythrocytic stage vaccine with full C-terminal $PvMSP1_{44}$ or immunodorminant $PvMSP1_{19}$ sequence alone by causing an additional protective effect or antibody maintenance.

적 요

삼일열 말라리아는 전세계적으로 가장 널리 분포하는 인체 감염 말라리아이다. 온대 지방에서 호발하는 재발과 재감염이 삼일열 말라리아의 가장 큰 특징이다. 오랜 노력에도 불구하고 아직 삼일열 말라리아에 대한 백신은 개발되지 않았다. 본 연구는 삼일열 원충(*Plasmodium viviax*) merozoite surface protein-1 (PvMSP1)의 33 kDa 분획을 발현하는 재조합 약독화 한국 균주(KVAC103)백시니아 바이러스 백신주를 제작하고 말라리아 백신으로서의 가치를 평가하고자 하였다. Vero 세포에서 PvMSP1의 33 kDa 분획을 발현하는 백신주(KVAC-PvMSP133)를 생산하고 정제하였다. 이 백신주를 3주 간격으로 마우스에 피하 접종하고 마우스의 세포성 및 혈액성 면역반응을 조사하였다. KVAC-PvMSP133 접종은 마우스에서 $CD3^+$, $CD4^+$, $CD8^+$ T 세포 반응을 일으켰으나 매우 미약하였다. 그러나 PvMSP1의 33 kDa 분획에 대한 IgG 항체는 크게 증가하였다. IgG 항체의 subtype 중 IgG2b가 IgG 항체 증가의 가장 큰 부분을 차지하였다. 전체적으로 보았을 때 KVAC-PvMSP133 접종은 마우스에서 세포성 면역보다 체액성 면역을 더 잘 유도하는 것으로 판단되었다. 이상의 결과는 KVAC103이 효과적인 항말라리아 백신 제작을 위한 플랫폼으로 유용함을 보여준다.

Acknowledgments

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Conflict of Interest

The authors have no conflict of interest related to this study.

Ethical Statement

This study was approved by the research ethic committee of Korea Centers for Disease Control and Prevention (KCDC-042-17-2A).

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