Evaluation of antibody responses to the early transcribed membrane protein family in *Plasmodium vivax*

Seong-Kyun Lee¹, Jin-Hee Han¹, Ji-Hoon Park¹, Kwon-Soo Ha², Won Sun Park³, Seok-Ho Hong⁴, Sunghun Na⁵, Yang Cheng⁶† and Eun-Taek Han¹†

**Abstract**

**Background:** Malaria parasites form intracellular membranes that separate the parasite from the internal space of erythrocytes, and membrane proteins from the parasites are exported to the host via the membrane. In our previous study, *Plasmodium vivax* early transcribed membrane protein (PvETRAMP) 11.2, an intracellular membrane protein that is highly expressed in blood-stage parasites, was characterized as a highly immunogenic protein in *P. vivax* malaria patients. However, the other PvETRAMP family proteins have not yet been investigated. In this study, PvETRAMPs were expressed and evaluated to determine their immunological profiles.

**Methods:** The protein structure and amino acid alignment were carried out using bioinformatics analysis software. A total of six PvETRAMP family proteins were successfully expressed and purified using a wheat germ cell free protein expression system and the purified proteins were used for protein microarray and immunization of mice. The localization of the protein was determined with serum against PvETRAMP4. IgG subclasses were assessed from the immunized mice.

**Results:** In silico analysis showed that *P. vivax* exhibits nine genes encoding the ETRAMP family. The ETRAMP family proteins are relatively small molecules with conserved structural features. A total of 6 recombinant ETRAMP proteins were successfully expressed and purified. The serum positivity of *P. vivax* malaria patients and healthy individuals was evaluated using a protein microarray method. Among the PvETRAMPs, ETRAMP4 showed the highest positivity rate of 62%, comparable to that of PvETRAMP11.2, which served as the positive control, and a typical export pattern of PvETRAMP4 was observed in the *P. vivax* parasite. The assessment of IgG subclasses in mice immunized with PvETRAMP4 showed high levels of IgG1 and IgG2b. PvETRAMP family proteins were identified and characterized as serological markers.

**Conclusions:** The relatively high antibody responses to PvETRAMP4 as well as the specific IgG subclasses observed in immunized mice suggest that the ETRAMP family is immunogenic in pathogens and can be used as a protein marker and for vaccine development.

**Keywords:** Malaria, *Plasmodium vivax*, ETRAMP, IgG antibody response, *P. vivax* patients

Correspondence: woerseng@126.com; ethan@kangwon.ac.kr

Yang Cheng and Eun-Taek Han contributed equally to this work

¹ Department of Medical Environmental Biology and Tropical Medicine, School of Medicine, Kangwon National University, Chuncheon, Gangwon-do 24341, Republic of Korea

⁶ Department of Public Health and Preventive Medicine, Laboratory of Pathogen Infection and Immunity, Wuxi School of Medicine, Jiangnan University, Wuxi, Jiangsu, People’s Republic of China

Full list of author information is available at the end of the article

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Background

Malaria is a major global public health problem and causes pathogen-specific mortality. Approximately three billion people from approximately 100 countries are exposed to the five human-infectious malaria parasites, resulting in 219 million malaria infections in 2017 [1, 2]. In order to eliminate the parasite from the host, malaria-specific antibodies play essential roles in acquired immunity via the phagocytosis and opsonization of parasites [3], and the importance of immunity is evident in experimental animal models as well as passive transfer studies in which antibodies from a parasite-infected donor patient have been used to effectively treat patients with severe malaria [4, 5]. Moreover, the identification and the evaluation of antibodies raised against unknown merozoite antigens involved in parasite survival is also necessary for the development of serological markers as well as a vaccine [6–8]. For this reason, the development of omics techniques and high-throughput screening systems involving cell-free protein synthesis technology has led to the identification of numerous vaccine candidates and has extended the possibilities for investigating serological markers that induce an immune response in endemic areas of Plasmodium falciparum and P. vivax [9–12].

The early transcribed membrane protein (ETRAMP) family was identified first in human-infectious malaria parasites, followed by rodent malaria parasites, and shows conserved structural features such as the presence of a signal peptide at the N terminus and a charged domain in the C-terminal region [13–17]. Interestingly, most ETRAMPs are expressed in stage-specific expression patterns during the parasite life cycle, and they mostly localize to the parasitophorous vacuole membrane (PVM), which spatially separates the parasite from the cytosol of erythrocytes in infected RBCs and mediates the free passage of molecules, probably through membraneous pores such as the Plasmodium transloco for exported proteins (PTEX) and exported protein 1 (EXP1) [18–22]. On the other hand, the P. berghei ETRAMP family member small exported protein 2 (SEP2), localizes to membranous compartments of the ookinete and is released during gliding motility in the sporozoite, indicating that the protein family is involved in not only blood stage but also sexual stage [23].

In our previous studies, 232 blood stage-specific proteins of P. vivax were screened using P. vivax patient serum samples to evaluate the immune response and explore vaccine candidates using a protein array method, and PvETRAMP11.2 showed a relatively high antibody responses under relapse and reinfection of P. vivax according to serological analysis, indicating that the PvETRAMP family is likely to be immunogenic in P. vivax patients [24–26]. Therefore, in the present study, the other ETRAMPs of P. vivax were expressed for evaluation of serum reactivity to P. vivax-infected patient sera and profiling of the IgG subclass phenotype.

Methods

Bioinformatics analysis

The genes encoding pvetramps were retrieved from Plasmodb (http://plasmodb.org), along with their predicted transcriptional expression levels in blood-stage parasites. Alignments of the corresponding amino acid sequences were carried out with Clustal W2 [27]. The Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) and the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/service/TMHMM) were used for the prediction of signal peptides and transmembrane domains [28–31].

Sample and serum preparation

Blood samples that were confirmed to be either P. vivax positive or negative via testing with a rapid diagnosis test kit and microscopy were collected in the Republic of Korea with approval from the Institutional Review Board at Kangwon National University (IRB No. 2017–05–009–001). The collected whole blood samples were centrifuged to separate the serum and packed cells.

Cloning and protein expression of PvETRAMPs

The six pvetramps were amplified by PCR from genomic DNA isolated from Korean P. vivax malaria patients. The primers used for PCR are described in Table 1. The six amplified genes were cloned into the Xhol and BamHII sites of the pEU-E01-His-Tev-N2 plasmid vector (Cell-Free Sciences, Matsuyama, Japan) for protein expression using in-fusion cloning (Clontech, Palo Alto, CA, USA). The nucleotide sequence of pvetramps in the pEU plasmid DNA vector was confirmed by sequence analysis (Genothech, Daejeon, Korea). The pEU plasmid DNAs were purified with a Midiplus ultrapure plasmid extraction system (Viogene, Taipei, Taiwan) and were used for protein expression as described in a previous report [32]. The recombinant proteins were purified using a Ninitrilotriacetic acid (Ni-NTA) affinity chromatography column (Qiagen, Hilden, Germany) [33]. The purified proteins were confirmed by 12% SDS-PAGE and western blot analysis under reducing conditions.

Evaluation of antibody responses against six recombinant PvETRAMPs in P. vivax malaria patients or healthy individuals using a protein microarray

The preparation of amine-coated slides was described in a previous report [25]. Serum samples from P. vivax malaria patients or healthy individuals were used to
evaluate the antibody reactivity of PvETRAMPs. A recombinant protein (100 ng in each protein) was spotted in each well of a slide, followed by incubation for 2 h at 37 °C. After washing the slide with phosphate-buffered saline-Tween (PBST, 0.1%), the slide was blocked with 5% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. The slide was probed with serum (1:10 dilution) and incubated for 1 h at 37 °C. Alexa Fluor 546-conjugated goat anti-human IgG (10 ng/μl; Invitrogen, Carlsbad, CA, USA) was spotted on the slide, followed by incubation for 1 h at 37 °C for visualization. The slide was scanned in a fluorescence scanner (Perkin Elmer, Boston, MA, USA) [34]. The mean fluorescence intensity (MFI) of each spot was quantified using ScanArray Express software version 4.0 (Perkin Elmer). The cut-off MFI value was determined as the MFI of serum from a healthy individual plus two standard deviations [33].

**Indirect immunofluorescence assay (IFA)**

Slides smeared with an enriched schizont-stage of *P. vivax* from patient blood samples were fixed with ice-cold acetone for 3 min, dried, and stored at −80 °C until use. The slides were blocked with 5% BSA in PBS at 37 °C for 30 min and washed with PBS. The slides were incubated with a 1:50 diluted primary antibody (mouse anti-PvETRAMP4 and rabbit anti-PvETRAMP11.2) at 37 °C for 1 h. The slides were washed with Alexa Fluor 488-conjugated anti-mouse IgG, Alexa Fluor 568-conjugated anti-mouse IgG and Alexa Fluor 568-conjugated goat anti-rabbit IgG secondary antibodies (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) at 37 °C for 30 min. The stained slides were mounted in ProLong Gold antifade reagent (Invitrogen) and visualized under a confocal laser scanning FV200 microscope (Olympus, Tokyo, Japan). The captured images were analyzed using FV10-ASW 3.0 viewer software.

**Immunization and IgG subclass profiling**

To induce IgGs against PvETRAMP4, 20 μg of recombinant protein was injected into 5-week-old female BALB/c mice (Daehan Biolink Co., Eumsung, Korea) with Freund’s complete adjuvant, followed by an incomplete adjuvant. The injections were performed a total of three times at 2-week intervals. Enzyme-linked immunosorbent assays (ELISAs) were used for the profiling of IgG subclasses produced against PvETRAMP4 in mice [35]. Ninety-six-well ELISA plates (Costar, Corning, NY) were coated with purified mouse IgG1, IgG2a, IgG2b, and IgG3 (Invitrogen) and incubated with immunized mouse sera diluted 1:1000 in PBST. Horse-radish peroxidase-conjugated anti-mouse IgG1, IgG2a, IgG2b, and IgG3 antibodies (Invitrogen) at 1:1000, 1:1000, 1:2000, and 1:1000 dilutions, respectively, were used to detect each isotype antibody. The reaction was developed by adding 100 μl of diluted 3, 3′, 5, 5′-tetramethylbenzidine single solution (Invitrogen, Rockford, IL) for 15 min at 37 °C, then stopped with 100 μl of 1N HCl solution. The intensity was measured and calculated from the log–log curve fit.

**Statistical analysis**

The data were analyzed with GraphPad Prism (GraphPad Software, San Diego, CA) and Microsoft Excel 2016 (Microsoft, Redmond, WA). Unpaired t-tests were used to compare the differences between the means of each group. Values of *P* <0.05 were considered statistically significant. Sensitivity was calculated based on the percentage of patients exhibiting values above the cut-off MFI, and specificity was calculated based on the percentage of healthy patients presenting values below the cut-off MFI.

### Table 1  Primer sequence information for pvetramps for cloning into the pEU expression vector

| Gene ID | ORF (bp) | Expression region sequence (bp) | Genic-specific sense primer (5′–3′) | Genic-specific anti-sense primer (5′–3′) |
|---------|---------|---------------------------------|-------------------------------------|---------------------------------------|
| PVX_090230 | 438 | 238–438 (201) | GGGCGCGATATCTCGAGTAGCTACACGAGAAGAAGAGCAAGG | GCGGTACCCGGGATTCCTACATTTGGATGGGGGTT |
| PVX_096070 | 549 | 79–627 (549) | GGGCGCGATATCTCGAGCATCTGAATACAAAGGCCCAAGC | GCGGTACCGGGGATTCCTTAAAGATTCTGTTGAGGTTGTTCT |
| PVX_086915 | 564 | 76–564 (489) | GGGCGCGATATCTCGAGAATTATTCTGAAAAAGTGAAAGC | GCGGTACCCGGGATACCTCATTTGTTTGGATGGGGT |
| PVX_088870 | 519 | 76–519 (444) | GGGCGCGATATCTCGAGGAGGATGTGTAAGAGAAGAAGAGAAGAGAAG | GCGGTACCGGGGATCCCTTAAAGATTCTGTTGAGGTTGTTCT |
| PVX_001715 | 504 | 64–501 (438) | GGGCGCGATATCTCGAGGAGGATGTGTAAGAGAAGAAGAGAAGAGAAG | ACGTCTCAGTATAATGAGAGAGACCAAGAAG |
| PVX_003565 | 333 | 67–333 (267) | GGGCGCGATATCTCGAGTTCTCAAAATATGGTTAGCAAGGAAAG | GCGGTACCGGGGATACCTCATTTGTTTGGATGGGGT |

Note: The vector sequences are shown in **bold**.
### Results

#### Identification of PvETRAMP proteins

Nine proteins in the ETRAMP family were found in *P. vivax*, and the proteins were named according to their orthologues in *P. falciparum*. The PvETRAMPs were relatively small molecules except for PvETRAMP10.2, showing sizes of 11.9–25.0 kDa and 110–212 amino acids (Table 2, Fig. 1a). However, all PvETRAMPs presented high conservation of a signal peptide at the N terminus and one or two transmembrane domain(s) flanked by highly charged domain(s) containing amino acids such as lysine (K) and aspartic acid (D), leading most PvETRMPs to exhibit high pI values (Fig. 1a, b). In the transcriptome analysis, PvETRAMP4, 9, and 10.2 were predicted to be highly transcribed at the late trophozoite and early schizont stages, and PvETRAMP13 and 14.2 showed high transcription levels at the late schizont stage, indicating that expression of PvETRAMPs is mostly stage specific during intraerythrocytic development (Fig. 1c).

#### Expression and purification of recombinant PvETRAMP proteins from a wheat germ cell-free system

A total of six PvETRAMP proteins were successfully expressed and purified using a wheat germ cell-free system, and their purity was confirmed by SDS-PAGE and western blot analyses (Fig. 2). The specific bands observed in the elution fraction indicated that the recombinant proteins were purified in a soluble form. However, the expression or solubility rate of PvETRAMP9 and 11.2 was considerably lower than those of the others. Two proteins, PvETRAMP5 and PvETRAMP10.3, had different banding patterns in the SDS-PAGE and immunoblot results, which was thought to be non-specific proteins co-expressed with target proteins. Most of the recombinant proteins were observed at slightly greater or lower sizes than their expected molecular weights, which might be due to the pI values of proteins, and these results were consistent with PfETRAMPs (Table 2) [13].

#### Humoral immune response evaluation of PvETRAMPs

A protein array method was exploited to evaluate the humoral immune responses against PvETRAMPs (Fig. 3). All PvETRAMPs showed high specificity (>95%), indicating that they can be used for differentiating infected and non-infected humans. There were significant antibody reactivity differences between patients and healthy individuals (*P* < 0.05) for most PvETRAMPs, except for PvETRAMP9 (*P* = 0.05) (Table 3). The sera from *P. vivax*-infected individuals to PvETRAMPs showed significantly higher mean fluorescence intensity (MFI) values than those from malaria-naïve individuals. The highest seropositivity was found for PvETRAMP11.2 (76.8%), followed by PvETRAMP4 (62%), which was used for further study.

#### Subcellular localization of PvETRAMP4 in blood-stage parasites

Mice were immunized with a recombinant PvETRAMP4 protein for further analysis, and the specific serum reacted with the *P. vivax* isolate (Fig. 4). Interestingly, PvETRAMP4 showed signal associated membrane and organelle in different stages, and PvETRAMP11.2 partially colocalized with PvETRAMP4.

#### Isotype prevalence of the antibody response in immunized mice

To evaluate isotype prevalence, an anti-PvETRAMP4 antibody from immunized mice was analyzed by ELISA. As shown in Fig. 5, the major components of the antibody response against PvETRAMP4 were IgG1

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**Table 2** The genetic information for the etramp family in Plasmodium vivax

| Gene        | PlasmoDB gene ID | SP | TM | kDa | ORF (bp) | pI     |
|-------------|------------------|----|----|-----|---------|--------|
| *etramp4*   | PVX_090230       | Y  | TM | 15.8| 438     | 10.8   |
| *etramp5*   | PVX_096070       | Y  | TM | 21.0| 627     | 7.3    |
| *etramp9*   | PVX_086915       | Y  | TM | 21.5| 564     | 10.3   |
| *etramp8*   | PVX_088870       | Y  | TM | 18.9| 519     | 9.6    |
| *etramp10.2*| PVX_111065       | Y  | TM | 78.9| 2226    | 4.1    |
| *etramp10.3*| PVX_001715       | Y  | TM | 18.2| 504     | 9.1    |
| *etramp2/etramp11.2* | PVX_003565 | Y  | TM | 11.9| 333     | 11.0   |
| *etramp13*  | PVX_121950       | Y  | TM | 25.0| 642     | 8.9    |
| *etramp14.2*| PVX_118680       | Y  | TM | 20.0| 528     | 9.9    |

**Abbreviations:** SP, signal peptide; TM, transmembrane; pI, isoelectric point; ORF, open reading frame
and IgG2b. There was no significant difference between the levels of IgG2a and IgG3.

Discussion

The ETRAMP family is distributed in all human-infectious malaria species as well as other mammalian malaria parasites, such as rodent and primate parasites. In addition, several ETRAMP family proteins are abundantly transcribed in the erythrocytic stages of *P. falciparum* and *P. vivax* [36, 37]; therefore, characterization and identification of the ETRAMP family are carried out for the development of serological markers and novel vaccine candidate discovery.

*Plasmodium vivax* has nine identifiable etramps genes, as shown in Table 2, and thus fewer than in *P. falciparum*, in which 13 ETRAMPs have been identified and found to be expressed at a specific stage [13]. Similar to *P. falciparum*, PvETRAMPs were predicted to be expressed specifically at the schizont and early ring stages. It is useful to identify the stage-specific proteins in the parasite lifecycle because they may play important and specific roles in parasite development. For example, the transcription levels of invasive proteins such as merozoite surface proteins were found to be sharply increased in the late schizont stage, indicating that the merozoites interact with erythrocytes through these proteins. In a previous report, PvETRAMP11.2 was considered to be an interaction partner of PvTRAg36.6, which is exported to the RBC membrane in transgenic *P. falciparum* and localizes to the apical ends of the free merozoites in *P. vivax*; additionally, the interaction between PvETRAMP11.2 and PvEXP1, which is considered to be essential for parasite growth, was confirmed [38, 39]. Moreover, the lower expression levels of four ETRAMPs found in Saimiri monkeys, to which the *P. vivax* parasite can infect, but without PvDBP-II binding, suggest that ETRAMPs expressed at a specific stage either directly or indirectly
affect the regulation of functional protein(s) necessary for parasite growth [40].

One of the critical factors for the discovery and evaluation of potential targets that induce a strong humoral immune response in parasite-infected patients is the use of appropriate screening methods. In our previous study, a large number of \( P. \) \textit{vivax} antigens were screened using a wheat germ cell free-expression system and protein array with \( P. \) \textit{vivax} patient sera and healthy sera. A high correlation between the protein array and ELISA for the screening of serum reactivity was validated; therefore, we used the protein array method to analyze the humoral immune response against the ETRAMP family in \( P. \) \textit{vivax} [41, 42]. Interestingly, PvETRAMP4 showed higher serum positivity than the other PvETRAMPs tested, although it does not seem to be directly exposed to the human immune system (Fig. 3, Table 3). Generally, the antigen that induces a humoral immune response in the host, such as EMP-1 or MSP-1, is a result of exposure to the immune system and is usually located on the erythrocyte or merozoite surface, which is continuously exposed to the human immune system. However, there is a possibility that some proteins within infected erythrocytes can also be exposed to the immune system upon lysis of the parasite after egress [43]. For a deeper understanding of the immune system response to PvETRAMP4, the IgG subclasses generated in immunized mice were evaluated. The IgG subclasses produced against PvETRAMP4 showed that IgG1 and IgG2b were predominantly produced. In a previous study, cytophilic isotypes (IgG1 and mainly IgG3) were found to be predominantly produced by \( P. \) \textit{vivax} infection; IgG2b in mice plays a similar role to IgG3 in humans [44, 45], suggesting that PvETRAMP4 probably induces IgG3 in the human immune system. However, the immune response to other PvETRAMPs could not be evaluated due to a limited amount of protein. Therefore, there is a need to further study other ETRAMP members of \( P. \) \textit{vivax}.

![Fig. 2 Recombinant protein expression and purification of PvETRAMPs in a wheat germ cell-free expression system. a SDS-PAGE of purified PvETRAMPs. Arrows indicate purified PvETRAMP proteins. b Western blot of PvETRAMPs with an anti-penta-His antibody. Abbreviations: M, protein marker; T, total fraction; S, soluble fraction; P, pellet fraction; Ft, flow-through; E, elution fraction.](image)
Conclusions

For the immunological profiling of the *P. vivax* ETRAMP protein family, six proteins from the nine ETRAMP family proteins of *P. vivax* were expressed and purified, after which they were used for evaluation of the humoral immune response in *P. vivax* patients and immunization in mice. Interestingly, PvETRAMP4 and 11.2 showed relatively high antibody responses in the sera of *P. vivax* malaria patients or healthy individuals from the ROK. *P*-values were calculated with unpaired t-tests. The bar indicates the mean plus SD.

Table 3 Prevalence, 95% confidence intervals, and mean fluorescence intensities of IgG responses to PvETRAMPs in *P. vivax* malaria patients and healthy individuals

| Antigen (*P. vivax*) | Sample (n) | No. of samples | Sensitivity (%) / Specificity (%) | 95% CI (%) | MFI | Cut-off value | t-value | P-value
|----------------------|------------|----------------|-----------------------------------|------------|-----|--------------|---------|-----|
|                      |            | Positive / Negative |                                    |            |     |              |         |     |
| ETRAMP4              | Patients (50) | 31 / 19         | 62.0 / 97.5                       | 48.2–74.1  | 10,713 ± 10,787 | 52,033 | 4.341 | < 0.0001 |
|                      | Healthy (40)  | 1 / 39           |                                    | 87.1–99.6  | 3276 ± 963.4 |        |       |         |
| ETRAMP5              | Patients (50) | 24 / 26         | 48.0 / 95.0                       | 34.8–61.5  | 6115 ± 5629 | 4579  | 3.877 | 0.0002  |
|                      | Healthy (40)  | 2 / 38           |                                    | 83.5–98.6  | 2620 ± 979.4 |        |       |         |
| ETRAMP8              | Patients (50) | 23 / 27         | 46.0 / 95.0                       | 32.8–59.6  | 6678 ± 6376 | 4589  | 3.600 | 0.0005  |
|                      | Healthy (40)  | 2 / 38           |                                    | 83.5–98.6  | 3023 ± 783.1 |        |       |         |
| ETRAMP9              | Patients (32) | 14 / 18         | 43.8 / 100                        | 28.2–60.7  | 4428 ± 2008 | 3968  | 2.015 | 0.05    |
|                      | Healthy (8)   | 0 / 8            |                                    | 67.6–100   | 2974 ± 497.2 |        |       |         |
| ETRAMP10.3           | Patients (50) | 20 / 30         | 40.0 / 97.5                       | 27.6–53.8  | 5880 ± 5540 | 4259  | 3.643 | 0.0005  |
|                      | Healthy (40)  | 1 / 39           |                                    | 87.1–99.6  | 2659 ± 799.8 |        |       |         |
| ETRAMP11.2           | Patients (56) | 43 / 13         | 76.8 / 97.5                       | 64.2–85.9  | 12,647 ± 3326 | 9733  | 10.39 | < 0.001 |
|                      | Healthy (40)  | 1 / 39           |                                    | 87.1–99.5  | 6811 ± 1461 |        |       |         |

*a* Cut-off, the mean fluorescence intensity of the malaria-naive samples plus 2 standard deviations

*b* *P*-value, the difference in the total IgG level for each antigen between *P. vivax* malaria patients and healthy individuals was calculated with the unpaired t-test. A *P*-value of < 0.05 was considered statistically significant

Abbreviations: CI, confidence interval; MFI, mean fluorescence intensity
P. vivax patients, and IgG isotype profiling showed that IgG1 and IgG2b were predominant in mice immunized with PvETRAMP4, suggesting that the PvETRAMPs might be candidates for vaccine and serological markers.

**Abbreviations**
ETRAMP: early transcribed membrane protein; SEP: small exported proteins; PCR: polymerase chain reaction; IgG: immunoglobulin G; PVM: parasitophorous vacuole membrane; RBC: red blood cell; PTEX: Plasmodium translocon for exported proteins (PTEX); EXP-1: exported protein-1; MSP-1: merozoite surface protein 1; EMP-1: erythrocyte membrane protein-1.

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**Authors’ contributions**
SKL and YC equally contributed to the design, analysis, completion, interpretation, and reporting of the study. JHP and JHH participated in one or more of the experiments. KSH, WSP, SHH, SHN, and ETH analyzed the data and interpreted the results. All authors read and approved the final manuscript.

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**Availability of data and materials**
The data supporting the conclusions of this article are included within the article.

**Ethics approval and consent to participate**
The animal experiment was approved by the Institutional Ethics Committee and followed the Ethical Guidelines for Animal Experiments of Kangwon National University, Chuncheon, Republic of Korea (KIACUC-16-0158).

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

**Author details**
1 Department of Medical Environmental Biology and Tropical Medicine, School of Medicine, Kangwon National University, Chuncheon, Gangwon-do 24341, Republic of Korea. 2 Department of Cellular and Molecular Biology, School of Medicine, Kangwon National University, Chuncheon, Gangwon-do 24341,
Republic of Korea. 3 Department of Physiology, School of Medicine, Kangwon National University, Chuncheon, Gangwon-do 24341, Republic of Korea. 4 Department of Internal Medicine, School of Medicine, Kangwon National University, Chuncheon, Gangwon-do 24341, Republic of Korea. 5 Department of Obstetrics and Gynecology, Kangwon National University Hospital, Chuncheon, Gangwon-do 24341, Republic of Korea. 6 Department of Public Health and Preventive Medicine, Laboratory of Pathogen Infection and Immunity, Wuxi School of Medicine, Jiangnan University, Wuxi, Jiangsu, People’s Republic of China.

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